Eur J Plant Pathol (2011) 130:511–519
DOI 10.1007/s10658-011-9770-x

Somatic embryogenesis efficiently eliminates viroid infections from grapevines

Giorgio Gambino · Beatriz Navarro · Rosalina Vallania · Ivana Gribaudo · Francesco Di Serio

Accepted: 2 March 2011 / Published online: 18 March 2011

Abstract Indirect somatic embryogenesis is effective at eliminating the most important viruses affecting grapevines. Accordingly, this technique was tested as a method for eradicating two widespread viroids, *Grapevine yellow speckle viroid* 1 (GYSVd-1) and *Hop stunt viroid* (HSVd), from four grapevine cultivars. Both viroids were detected by RT-PCR in grapevine floral explants used for initiating embryogenic cultures, as well as in undifferentiated cells of embryogenic and non-embryogenic calli from anthers and ovaries. In contrast, somatic embryos differentiated from these infected calli were viroid-free, and viroids were not detected in embryo-derived plantlets even 3 years after their transfer to greenhouse conditions. A wider spatial distribution of HSVd than GYSVd-1 within proliferating calli was revealed by in situ hybridization, whereas no hybridization signal was detected in the somatic embryos. In addition, GYSVd-1 and HSVd were localised in the nucleus of infected cells, conclusively showing the nuclear accumulation of representative members of *Apscaviroid* and *Hostuviroid* genera, which has been only an assumption so far. Somatic embryogenesis was compared to in vitro thermotherapy, a technique routinely used for virus eradication. After thermotherapy, HSVd and GYSVd-1 were detected in all in vitro plantlets of the cultivar Roussan, and in all lines analysed after 3 years of culture in greenhouse. The high efficiency with which somatic embryogenesis may eliminate viroids and viruses from several infected grapevine cultivars, should allow the availability of virus- and viroid-free material, which would be useful not only for sanitary selection but also for basic research on plant-virus and plant-viroid interactions in grapevine.

Keywords HSVd · GYSVd-1 · *Vitis vinifera* · In situ hybridization · Thermotherapy · Nuclear localization

G. Gambino · R. Vallania · I. Gribaudo (✉)
Istituto di Virologia Vegetale, UOS Grugliasco,
Consiglio Nazionale delle Ricerche,
Via L. da Vinci 44,
10095 Grugliasco, TO, Italy
e-mail: i.gribaudo@ivv.cnr.it

B. Navarro · F. Di Serio (✉)
Istituto di Virologia Vegetale, UOS Bari,
Consiglio Nazionale delle Ricerche,
Via Amendola 165/A,
70126 Bari, Italy
e-mail: f.diserio@ba.ivv.cnr.it

Introduction

Viroids are small, circular, non-protein-coding RNAs infecting higher plants, which, in turn, may develop symptoms, resulting in economic losses in several crops (reviewed by Flores et al. 2005; Ding 2009). Most viroids are grouped within the family *Pospiviroidae* (type species *Potato spindle tuber viroid*, PSTVd), which includes viroid species with a genomic RNA assuming a rod-like or quasi rod-like secondary structure that contains a central conserved

region (CCR) and other structural elements used to allocate each species within five genera (*Pospiviroid, Apscaviroid, Hostuviroid, Cocadviroid* and *Coleoviroid*). Experimental data support the general view that all *Pospiviroidae* replicate and accumulate in the nucleus (reviewed by Flores et al. 2005), although conclusive data in this respect is still lacking for representative species of several genera within the family. Four additional viroid species, in which genomic RNA assumes a rod-like or branched conformation, lacks a CCR, and contains hammerhead ribozymes, have been grouped within the family *Avsunviroidae,* whose representative members replicate and accumulate within the chloroplast (reviewed by Flores et al. 2000).

Grapevine is one of the most permissive, natural viroid hosts. Five *Pospiviroidae* species, belonging to three different genera, have been isolated from grapevine plants (reviewed by Little and Rezaian 2003). *Grapevine yellow speckle viroid 1* (GYSVd-1) (Koltunow and Rezaian 1988) and *Grapevine yellow speckle viroid 2* (GYSVd-2) (Koltunow et al. 1989), which are two members of the genus *Apscaviroid,* have been identified as independent causal agents of yellow speckle disease, which is characterised by highly visible, yellow blotches on the grape leaves (Koltunow et al. 1989). Although yellow speckle disease significantly reduces the total healthy leaf area and occasionally affects the yield quality of grapevines in South Australia (Little and Rezaian 2003), these symptoms rarely appear in other geographical areas, most likely because they are strictly dependent on environmental conditions. GYSVd-1, and possibly GYSVd-2, in association with the *Grapevine fanleaf virus* (GFLV) have also been implicated in the elicitation of vein-binding, a common disease in both Europe and California, characterised by yellow mottling or banding along the principal leaf veins of affected grapevines (Szychowski et al. 1995). In contrast, no symptoms have been associated with natural grapevine infections by *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd) and *Australian grapevine viroid* (AGVd), members of the *Hostuviroid, Pospiviroid* and *Apscaviroid* genera, respectively (Sano et al. 1985; Garcia-Arenal et al. 1987; Rezaian 1990).

Latent grapevine infections can also be detrimental. In fact, symptomless infected plants may serve as source of inoculum for other susceptible crops, as recently shown in Japan, where HSVd outbreaks and subsequent epidemics in hop crops have been very likely initiated by HSVd infected grapevines which did not display any symptom (Kawaguchi-Ito et al. 2009). A similar scenario has been previously proposed for several citrus viroids, including HSVd and CEVd, whose primary inoculum source was hypothesised to be symptomless, viroid-infected grapevines grown in the Mediterranean basin and Near-East when the first citrus varieties were imported more than 1500 years ago in this geographic area (Bar-Joseph 1996).

Vegetative propagation and grafting practices largely favour worldwide dissemination of grapevine viroids. Similarly to viruses, the most effective strategy for preventing or reducing the spread of viroids relies on the use of pathogen-free propagation material. Meristem tip culture and thermotherapy are traditional techniques used to eliminate grapevine phloem-limited viruses and viruses that readily invade plant meristems, respectively. Unfortunately, the effectiveness of these techniques in eliminating viroids from infected grapevines seems poor. Although successful elimination of viroids from grapevines by shoot apical meristem (SAM) culture has been reported (Duran-Vila et al. 1988), the efficacy of this technique appears to be very low, as indicated by results when a highly sensitive molecular assay was used for viroid detection in resulting plantlets (Wan Chow Wah and Symon, 1997). Moreover, our own preliminary data indicates that thermotherapy is largely ineffective at eliminating viroids from infected grapevines.

Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programs, has been shown to efficiently eliminate several phloem-limited viruses (Goussard et al. 1991; Gambino et al. 2006) and two nepoviruses (Borroto-Fernandez et al. 2009; Gambino et al. 2009) from grapevines. In contrast, data on the efficiency of this technique for eradicating viroids from infected hosts is lacking. In this work, we report that somatic embryogenesis can eliminate viroids from several infected grapevine cultivars with high efficiency. Incidentally, to the best of our knowledge, this study also provides the first in situ hybridization-based evidence that viroid nuclear localization, which has been previously shown for PSTVd, can be extended to a representative member of the genus *Apscaviroid* and to HSVd, the unique member of the genus *Hostuviroid.*

Materials and methods

Plant material The following four Italian cultivars of *Vitis vinifera* L. were used for this study: Cari, Provinè, Roussan and Nebbiolo. Mother plants were tested for the presence of GYSVd-1 and HSVd (see below) by RT-PCR and their viral status was also analysed by multiplex RT-PCR (Gambino and Gribaudo 2006).

Somatic embryogenesis Embryogenic cultures were initiated from developing flowers of Cari, Provinè, Roussan and Nebbiolo collected in vineyard at the early stage corresponding to pollen mother cells in pre-meiotic phase. Stamens (anthers plus filaments) and pistils (ovaries plus styles, stigmas and receptacles) were isolated from inflorescences and indirect somatic embryogenesis was induced as previously described (Gribaudo et al. 2004). Below we refer to these explants simply as anthers and ovaries. Floral explants were cultivated on a callus induction medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid and 8.9 μM benzyl-amino-purine (BAP). After 3 months, calli were transferred to an embryo differentiation medium containing 10 μM 2-napthoxyacetic acid, 1 μM BAP, 20 μM filter sterilised indole-3-acetic acid, and 0.25% activated charcoal. Cultures were kept at 26°C in the dark. Somatic embryos were isolated from embryogenic calli and transferred to a medium without plant growth regulators (PGRs) for further growth and germination.

In vitro thermotherapy One month-old rooted Roussan plantlets, grown on a modified Murashige and Skoog (1962) medium with half-strength mineral salts and 20 g l^{-1} sucrose (MMS) without PGRs, were heat-treated in a culture room at 34 °C with a 16 h photoperiod and a total energy of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Plantlets were treated by thermotherapy for 40 to a maximum of 99 days; therapy was stopped when plantlets showed clear signs of stress. At the end of thermotherapy, the apical bud (about 2 mm) and the 2–3 axillary buds closest to the apex were excised and cultivated on MMS medium containing 3 μM BAP. Shoots were rooted by culturing them for 7 days on a rooting medium (MMS containing 2.5 μM indole-butyric acid and 2.5 μM α-naphtalene-acetic acid) and subsequently on an MMS medium without PGRs.

Single plantlets, derived from thermotherapy or somatic embryogenesis, were micropropagated by repeated subculturing of apical cuttings (3–4 cm long) on MMS medium without PGRs, thus giving rise to individual lines. In spring, plants were acclimatised and transferred to a cool greenhouse. The greenhouse received minimal heating during winter, so the lignified plants could enter dormancy.

Detection of viroids Leaves from mother plants, anthers and ovaries, embryogenic and non-embryogenic calli, somatic embryos and plants were tested by RT-PCR for the presence of GYSVd-1 and HSVd. Anthers and ovaries were sampled (20 mg) at culture initiation. Whole embryogenic and non-embryogenic calli (10 to 50 mg), originating from single anthers or ovaries, were collected 4 months after culture initiation. Single somatic embryos were sampled 6 to 8 months after culture initiation. Plantlets, obtained from somatic embryogenesis or thermotherapy, were sampled (200 mg) during micropropagation. In addition, plants from the same lines were tested after growing in a cool greenhouse for 1 and 3 years, by sampling phloem scraped from mature canes collected during winter pruning.

Total RNA was extracted following the protocol of Gambino et al. (2008). First-strand cDNA synthesis was performed as previously described (Gambino and Gribaudo 2006). Specific primers, reported by Eiras et al. (2006) and Polivka et al. (1996), were used to amplify GYSVd-1 and HSVd cDNAs, respectively. The PCR reaction mix (20 μl) contained 1 μl of cDNA, 0.2 mM of dNTPs, 0.25 μM of each primer, 1.5 mM of $MgCl_2$ and 0.5 units of Taq polymerase (PlatinumTaq polymerase, Invitrogen). PCR was performed for 30 cycles at 94 ° C for 30 s, 55 ° C for 30 s and 72 ° C for 30 s. For each sample, a control PCR amplification of *Vitis* 18 S rRNA was performed using specific primers (Gambino and Gribaudo 2006), in order to check for possible RT-PCR inhibitors or RNA degradation. Reaction products were analysed on 2% agarose gels buffered in TBE (45 mM Tris-borate, 1 mM EDTA) and visualised by UV-light after staining with ethidium bromide.

In situ hybridization Embryogenic 'Provinè' calli were collected 4 months after culture initiation. Paraffin-embedding was performed as previously described (Gambino et al. 2010). Probes for detecting

the plus strands of GYSVd-1 and HSVd were generated by in vitro transcription from previously described plasmid templates (Navarro et al. 2009) using the Digoxigenin RNA labelling kit (Roche Applied Science).

Hybridization and immunological detection were performed as described by Mayer et al. (1998), with some modifications. Briefly, sections were de-paraffinised in xylene and re-hydrated with an ethanol series, incubated for 30 min in proteinase K (1 μg/ml in 100 mM Tris–HCl, and 5 mM EDTA pH 8.0) at 37°C, 5 min in 4% paraformaldehyde at 4 °C, and 10 min in 0.1 M triethanolamine containing 0.25% v/v acetic anhydride. Sections were then dehydrated in an ethanol series, and pre-hybridised for 10 min at 45°C in 4X SSC (0.6 M NaCl, 60 mM sodium citrate) and 50% (*v*/*v*) deionised formamide. Hybridization was carried out overnight at 55°C in hybridization buffer (1X Denhardt's solution, 10% dextran sulphate, 10 mM DTT, 4X SSC, 1 mg/ml yeast tRNA, 1 mg/ml denatured and sheared salmon sperm DNA and 50% deionised formamide) containing 500 ng of probes per ml of solution. After hybridization, slides were washed 3 times at 55°C in 0.2 X SSC 0.1% SDS for 40 min. Probes were detected using the chromogenic substrates, nitroblue tetrazolium and 5-bromo-4-cloro-3-indolyl phosphate (NPT/BCIP), resulting in a blue precipitate that was visible under a light microscope.

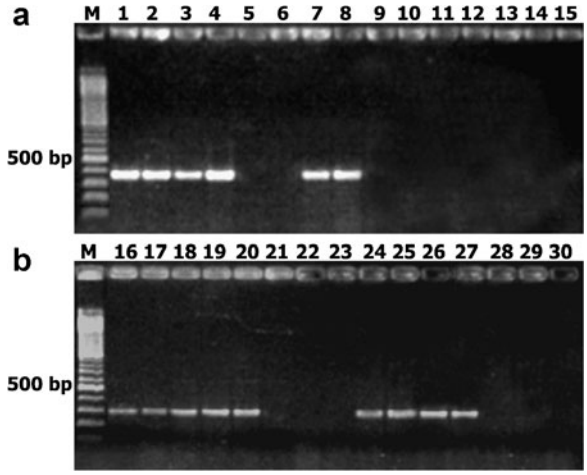

Fig. 1 Agarose gel analyses of RT-PCR assays with primers specific for GYSVd-1 (panel a) and HSVd (panel b) performed on representative samples of cv Provinè (panel a) and Roussan (panel b) respectively. Panel a: lane 1, leaves; lane 2, anthers; lane 3, ovaries; lanes 4–5, embryogenic calli from anthers; lanes 6–8, non embryogenic calli from anthers; lanes 9–10, somatic embryos; lanes 11–12, in vitro-cultured plantlets from somatic embryos; lanes 13–14, 3 year-old greenhouse-grown plants from somatic embryos. Panel b: lane 16, leaves; lane 17, anthers; lane 18, ovaries; lanes 19–20, in vitro-cultured plantlets originated from thermotherapy; lanes 21–23, in vitro-cultured plantlets from somatic embryos; lanes 24–27, 3 year-old greenhouse-grown plants originated from thermotherapy; lanes 28–29; 3 year-old greenhouse-grown plants from somatic embryos. Lanes 15 and 30, water control; M, 100 bp DNA ladder

Results

Identification and localization of HSVd and GYSVd-1 in mother plants, flower organs and differentiating calli

All tested mother plants of the grapevine cultivars Cari, Provinè, Roussan and Nebbiolo were infected by HSVd and GYSVd-1 (Fig. 1) in mixed infection with GFLV (Gambino et al. 2009). Therefore, they were considered to be suitable source material for testing the efficiency of somatic embryogenesis to eliminate viroids from infected grapevines.

First, we investigated whether HSVd and GYSVd-1 have infected the grapevine floral organs generally used for initiating embryogenic cultures. RNA preparations from anthers and ovaries, collected from Cari, Provinè and Roussan cultivar plants, tested positive by RT-PCR assays specific for HSVd and GYSVd-1 (Fig. 1). These data demonstrate that flower explants used for embryogenic cultures were viroid-infected and indicate that both viroids can infect flower organs at early developmental stages.

The presence and distribution of HSVd and GYSVd-1 were then investigated within calli at various stages of somatic embryogenesis. Due to the low embryogenesis efficiency of Cari, Roussan (Gambino et al. 2009) and Nebbiolo cultivars, we limited this study to Provinè.

After 4 months of culture, RT-PCR assays showed a high incidence of both HSVd and GYSVd-1 infections in embryogenic and non-embryogenic calli from anthers and ovaries (Fig. 1 and Table 1), indicating that both viroids are able to survive in undifferentiated calli cells. However, somatic embryos differentiated from these calli were viroid-free, suggesting that both HSVd and GYSVd-1 are excluded from the callus-derived embryos at an early stage of their differentiation (Fig. 1 and Table 1).

Table 1 Incidence of viroid infections during somatic embryogenesis from explants of cv Provinè

Callus type	GYSVd-1[a]	HSVd[a]
Non embryogenic from anthers	6/8	6/8
Non embryogenic from ovaries	9/10	10/10
Embryogenic from anthers	3/5	3/5
Somatic embryos	0/9	0/9

[a] Number of infected/number of tested samples

The spatial distribution of both viroids within proliferating calli was investigated by in situ hybridization. A deep blue color, revealing the presence of the target RNAs, was detected in serial sections of pre-embryonic masses hybridized with full-length RNA probes specific for HSVd and GYSVd-1 (Fig. 2). The localization of positive hybridization signals within the cells supports the proposed nuclear localization of these viroids (Fig. 2b and d). GYSVd-1 infected cells were found to be especially located in the external region of proliferating calli, whereas HSVd infected cells were observed over a wider distribution within the calli (Fig. 2a and c). As expected, on the basis of RT-PCR analyses, only HSVd-free and GYSVd-1-free somatic embryos were identified within the infected calli (Fig. 3), confirming the elimination of both viroids in the differentiated embryos.

Detailed histological observations of embryogenic cultures have been reported elsewhere (Gambino et al. 2010). However, it could be of interest here to remark that no vascular connection between embryoids or between grapevine somatic embryos and the parent tissues was observed.

Efficient elimination of viroids by somatic embryogenesis

Plantlets, regenerated by somatic embryogenesis from all of the cultivars tested (Cari, Provinè, Roussan and Nebbiolo), were sampled during in vitro micropropagation and tested by RT-PCR for viroid infections. As shown in Table 2, all 83 plantlets from the 4 cultivars assayed were found to be viroid-free (see also Fig. 1). To exclude the possibility that these negative RT-PCR results could be due to low viroid titres in plantlets grown under in vitro conditions, we extended our analyses to 20 embryo-derived Provinè, Roussan and Cari cultivar plants, which had been grown in a

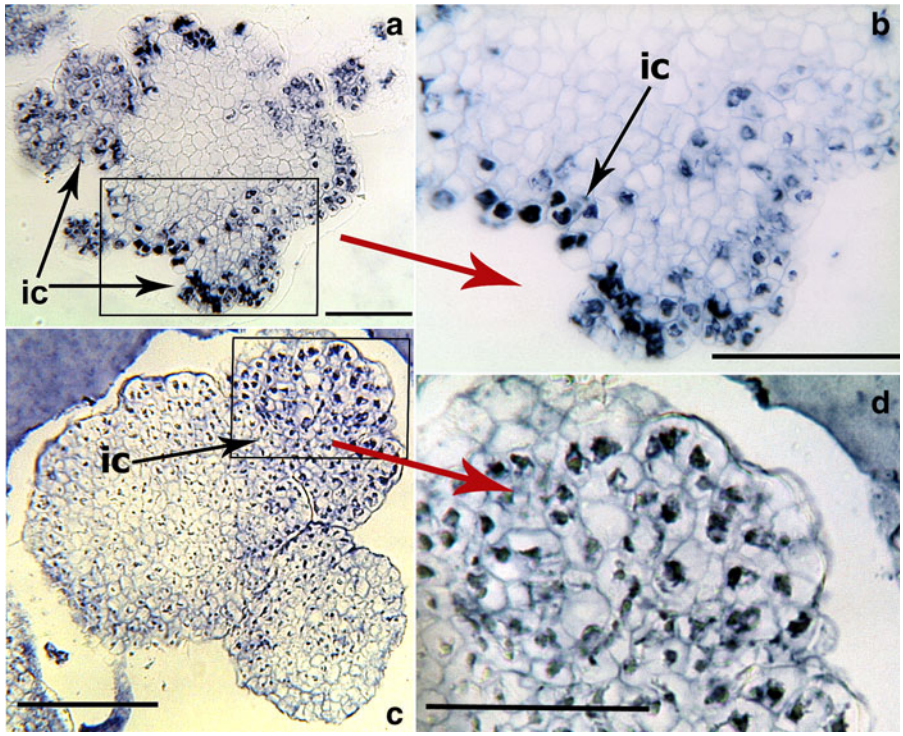

Fig. 2 In situ hybridization detection of GYSVd-1 and HSVd in embryogenic calli of cv Provinè 4 months after culture initiation. The intense blue color inside the cells indicates the presence of target viroid RNAs within the nuclei. Pre-embryogenic masses with GYSVd-1 (panel a) and HSVd-infected cells (panel c) are indicated by black arrows. The area inside the boxes in panels a and b are enlarged in panels c and d to show a closer view of GYSVd-1 and HSVd infected cells, respectively. ic, infected callus tissues. Size bar=100 μm

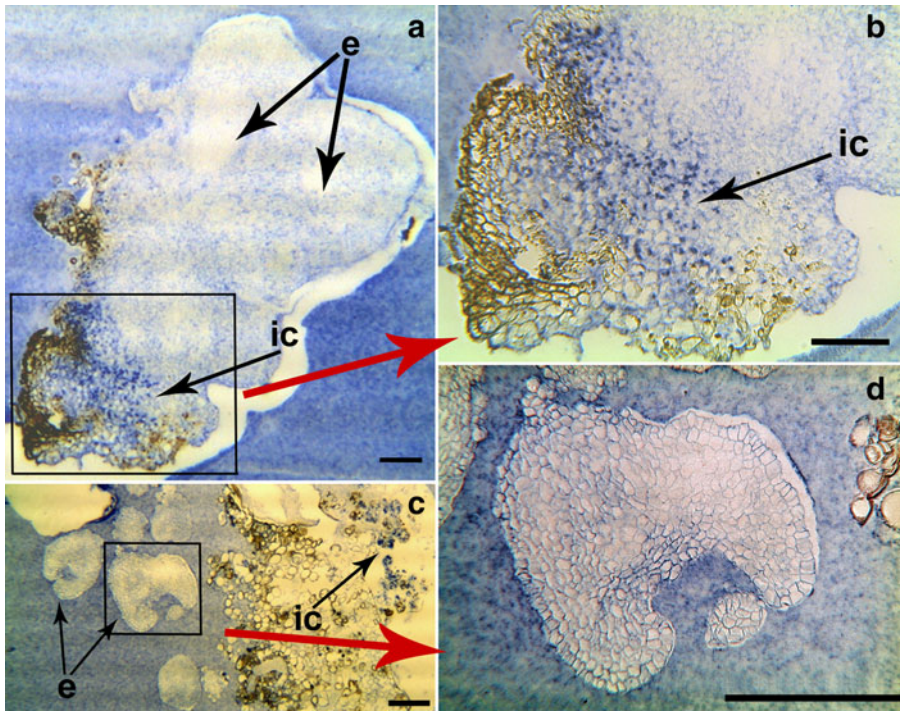

Fig. 3 In situ hybridization detection of GYSVd-1 and HSVd in somatic embryos and embryogenic calli of Provinè 4 months after culture initiation. The intense blue colour inside the cells indicates the presence of target viroid RNAs within the nuclei. Panel a, HSVd-free somatic embryos developing from infected callus; the area of HSVd-infected callus is enlarged in the panel b. Panel c, GYSVd-1-free somatic embryos localized near to infected callus cells; viroid-free heart-stage embryo is enlarged in panel d. ic, infected callus tissues; e, somatic embryos. Size bar=100 μm

greenhouse for a period of up to 3 years. All of the tested plants were found to be viroid-free (Table 2), independent of cultivar, indicating that somatic embryogenesis had a sanitation efficiency of 100%.

Low efficiency of viroid elimination by in vitro thermotherapy

In the case of cv Roussan, the sanitation efficiency of somatic embryogenesis was compared to that of thermotherapy; a technique routinely used for the eradication of viruses able to invade meristems. A total of 34 independent lines were produced following thermotherapy. HSVd and GYSVd-1 viroids were detected in all in vitro plantlets, and in all lines analysed after 3 years of greenhouse growth (Table 2 and Fig. 1). However, the same plants were found to be free of GFLV (data not shown), which was originally present in the mother plants. Thus, these results show that thermotherapy is ineffective at

Table 2 Incidence of viroid infections in plants generated by somatic embryogenesis (SE) or thermotherapy (T)

Cultivar	Sanitation method	Tested plant	GYSVd-1[a]	HSVd[a]
Provinè	SE	In vitro	0/33	0/33
	SE	1-year-old	0/1	0/1
	SE	3-year-old	0/8	0/8
Cari	SE	In vitro	0/16	0/16
	SE	1-year-old	0/7	0/7
	SE	3-year-old	0/10	0/10
Roussan	SE	In vitro	0/13	0/13
	SE	1-year-old	0/2	0/2
	SE	3-year-old	0/2	0/2
	T	In vitro	34/34	34/34
	T	3-year-old	4/4	4/4
Nebbiolo	SE	In vitro	0/21	0/21

[a] Number of infected/ number of tested samples

eliminating viroids from infected grapevines, while confirming its effectiveness against GFLV.

Discussion

Grapevine viroids are widespread and generally co-exist in mixed infections. In fact, many commercialised, virus-free vine cultivars and clones are infected by viroids, thus enabling the spread of these infectious agents. Although thermotherapy and/or SAM culture can eliminate many viruses from infected grapevines, their efficacy in eliminating viroids is questionable. This work shows, for the first time, that somatic embryogenesis is a highly effective technique for viroid elimination from infected grapevines. Four different grapevine cultivars were tested, and all of the embryo-generated plants were found to be viroid-free, suggesting that the sanitation efficiency of somatic embryogenesis is both extremely high and cultivar independent.

This technique has also some constraints, such as the possibility of somaclonal variation induction and the strong genotype-dependence, which results in particularly low or even null embryogenesis for some cultivars under the culture conditions tested (see Martinelli and Gribaudo 2009). Nonetheless, although the Nebbiolo, Cari and Roussan grapevine cultivars used in this study have low embryogenesis efficiency, the high sanitation efficiency demonstrated for somatic embryogenesis ensures that the few regenerated plants would be viroid-free. In contrast, thermotherapy treatment applied to Roussan cultivar was ineffective against viroid infections. These findings support our preliminary results, which indicate that most grapevine cultivars sanitised of viruses by either thermotherapy or SAM culture are still infected by viroids (data not shown).

In this study, HSVd and GYSVd-1 were detected by RT-PCR and in situ hybridization in calli from infected mother plants after 4 months of culture, indicating that these viroids may survive in undifferentiated cells. These findings suggest that both viroids have the potentiality of surviving also within the undifferentiated meristematic cells of infected plants; this possibility would also be in line with the apparent difficulty of eliminating viroids from infected grapevine by SAM culture (Wan Chow Wah and Symons 1997). However, direct experimental tests are needed to ascertain whether or not HSVd and GYSVd-1 actually enter grapevine meristems.

Interestingly, the two viroids appear to be differentially distributed within the infected calli. Differential distribution within the calli has also been previously reported for GFLV and two phloem-limited viruses and was proposed to be related to mechanisms of viral movement in tissues, possibly due to the characteristics and evolution of the callus after several months of culture (Gambino et al. 2010). However, factors leading to the differential distribution of HSVd and GYSVd-1 observed here remain unknown, and additional studies are needed to investigate the distribution and movement of these viroids within infected plants and to formulate specific hypotheses. Although both viroids are excluded from somatic embryos during embryogenesis, our results did not allow us to ascertain whether viroid sanitation is due to generalised impairment of viroid translocation within the calli, or to some alternative mechanism, such as possible impairment of embryogenesis in viroid-infected cells and/or the involvement of a defence mechanism specifically targeting invading RNAs in embryo-generating cells. Interestingly, it has been recently shown that PSTVd exclusion from SAM (Zhu et al. 2001; Qi and Ding 2003) is likely due to an RNA silencing-based degradation mechanism (Di Serio et al. 2010).

In situ hybridization results indicate that GYSVd-1 and HSVd localise to the nucleus of infected cells. To the best of our knowledge, this is the first direct evidence that a member of the *Apscaviroid* and *Hostuviroid* genera can accumulate in the nucleus. The generally accepted hypothesis that all *Pospiviroida*e accumulate within the nucleus is primarily based on structural and biochemical similarities between some members of the genera *Pospiviroid* (PSTVd and CEVd) and *Cocadviroid*, whose nuclear localization has been extensively studied (reviewed by Flores et al. 2005; Ding 2009), and viroids belonging to other genera within the family. Our results now provide experimental evidence in support of this hypothesis for two additional genera. The efficient elimination of GYSVd-1 and HSVd from grapevine infected tissues by somatic embryogenesis does not seem to be directly linked to their nuclear accumulation. In fact, the same technique has been successfully used for several grapevine cultivars to eradicate many cytoplasm-localized

viruses (Goussard et al. 1991; Gambino et al. 2006; Borroto-Fernandez et al. 2009; Gambino et al. 2009), including the *Grapevine rupestris stem pitting-associated virus*, for which the somatic embryogenesis seems to be the only reliable sanitation method (Gribaudo et al. 2006).

In conclusion, the somatic embryogenesis technique appears potentially suitable to concurrently eliminate most virus- and viroid-like disease agents from grapevine. The availability of this virus and viroid-free material would be useful not only for the nursery, but also for research on the etiology of several virus-like diseases, because pure preparations of the viroid(s) and/or virus(es) of interest can be inoculated into such healthy plants in single and mixed infections, overcoming the risk of synergistic or antagonistic effects of concurrent and unwanted viruses and/or viroids infecting the cultivars or clones to be tested. For a similar reason, the availability of this material will supply healthy controls for further dissecting the molecular basis of plant-viroid interactions in grapevines, which, to date, has been largely limited because of the difficulty of finding viroid-free material. Although most viroids do not induce visible symptoms in infected grapevines, viroid interference with the regulation of host gene expression and metabolic pathways, and their influence on the quality and/or quantity of the final crop, have not been thoroughly investigated. The availability of virus- and viroid-free grapevine cultivars, made possible by somatic embryogenesis, opens up new and fascinating perspectives that have been further enhanced by the publication of the complete grapevine genome sequence (Jaillon et al. 2007; Velasco et al. 2007) and by the recent application of genome-wide technologies to grapevines (Navarro et al. 2009; Mica et al. 2009; Pantaleo et al. 2010). Altogether, these factors contribute to making grapevines one of the best available experimental models for exploring the molecular mechanisms underlying plant-viroid interactions by high-throughput technologies. Finally, the possibility of using viroid-free material should be also considered for investigations into grapevine responses to other biotic and abiotic stresses, because infections by viroids or viroid mixtures could compromise the reproducibility of the experiments, especially if different viroids are present in the tested samples.

Acknowledgements Our thanks go to A. Schneider and F. Mannini for providing the mother plants. This research was partly funded by Regione Piemonte (Tech4Wine Project) and by the Dipartimento Agroalimentare of the CNR of Italy (A. Leone and D. Mariotti 2008 Award for Advanced Research in Agriculture to the last author).

References

Bar-Joseph, M. (1996). Natural history of viroids—Horticultural aspects. In A. Hadidi, R. Flores, J. W. Randles, & J. S. Semancik (Eds.), *Viroids* (pp. 246–251). Collingwood: CSIRO Publishing.

Borroto-Fernandez, E. G., Sommerbauer, T., Popowich, E., Schartl, A., & Laimer, M. (2009). Somatic embryogenesis from anthers of the autochthonous *Vitis vinifera* cv. Domina leads to Arabis mosaic virus-free plants. *European Journal of Plant Pathology, 124*, 171–174.

Di Serio, F., Martínez de Alba, A. E., Navarro, B., Gisel, A., & Flores, R. (2010). RNA-dependent RNA polymerase 6 delays accumulation and precludes meristem invasion of a nuclear-replicating viroid. *Journal of Virology, 84*, 2477–2489.

Ding, B. (2009). The biology of viroid-host interactions. *Annual Review of Phytopathology, 47*, 105–131.

Duran-Vila, N., Juarez, J., & Arregui, J. M. (1988). Production of viroid-free grapevines by shoot tip culture. *American Journal of Enology and Viticulture, 39*, 217–220.

Eiras, M., Targon, M. L. P. N., Fajardo, T. V. M., Flores, R., & Kitajima, E. W. (2006). *Citrus exocortis viroid* and *Hop stunt viroid* doubly infecting grapevine in Brazil. *Fitopatologia Brasileira, 31*, 440–444.

Flores, R., Daros, J. A., & Hernández, C. (2000). The *Avsunviroidae* family: viroids with hammerhead ribozymes. *Advances in Virus Research, 55*, 271–323.

Flores, R., Hernández, C., Martínez de Alba, A. E., Daros, J. A., & Di Serio, F. (2005). Viroids and viroid–host interactions. *Annual Review of Phytopathology, 43*, 117–139.

Gambino, G., & Gribaudo, I. (2006). Simultaneous detection of nine grapevine viruses by multiplex RT-PCR with coamplification of a plant RNA internal control. *Phytopathology, 96*, 1223–1229.

Gambino, G., Bondaz, J., & Gribaudo, I. (2006). Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. *European Journal of Plant Pathology, 114*, 397–404.

Gambino, G., Perrone, I., & Gribaudo, I. (2008). A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis, 19*, 520–525.

Gambino, G., Di Matteo, D., & Gribaudo, I. (2009). Elimination of *Grapevine fanleaf virus* from three *Vitis vinifera* cultivars by somatic embryogenesis. *European Journal of Plant Pathology, 123*, 57–60.

Gambino, G., Vallania, R., & Gribaudo, I. (2010). *In situ* localization of *Grapevine fanleaf virus* and phloem-restricted viruses in embryogenic callus of *Vitis vinifera*. *European Journal of Plant Pathology, 127*, 557–570.

Garcia-Arenal, F., Pallas, V., & Flores, R. (1987). The sequence of a viroid from grapevine closely related to severe isolates of *Citrus exocortis viroid*. *Nucleic Acids Research, 15*, 4203–4210.

Goussard, P. G., Wiid, J., & Kasdorf, G. G. F. (1991). The effectiveness of in vitro somatic embryogenesis in eliminating fanleaf virus and leafroll associated viruses from grapevines. *South African Journal of Enology and Viticulture, 12*, 77–81.

Gribaudo, I., Gambino, G., & Vallania, R. (2004). Somatic embryogenesis from grapevine anthers: identification of the optimal developmental stage for collecting explants. *American Journal of Enology and Viticulture, 55*, 427–430.

Gribaudo, I., Gambino, G., Cuozzo, D., & Mannini, F. (2006). Attempts to eliminate *Grapevine rupestris stem pitting-associated virus* from grapevine clones. *Journal of Plant Pathology, 88*, 293–298.

Jaillon, O., Aury, J. M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., et al. (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature, 449*, 463–467.

Kawaguchi-Ito, Y., Li, S. F., Tagawa, M., Araki, H., Goshono, M., Yamamoto, S., et al. (2009). Cultivated grapevines represent a symptomless reservoir for the transmission of *Hop Stunt Viroid* to hop crops: 15 years of evolutionary analysis. *PLoS ONE, 4*, e8386. doi:10.1371/journal.pone.0008386. http://www.plosone.org/home.action.

Koltunow, A. M., Krake, L. R., Johnson, S. D., & Rezaian, M. A. (1989). Two related viroids cause grapevine yellow speckle disease independently. *Journal of General Virology, 70*, 3411–3419.

Koltunow, A. M., & Rezaian, M. A. (1988). *Grapevine yellow speckle viroid*: structural features of a new viroid group. *Nucleic Acid Research, 16*, 849–864.

Little, A., & Rezaian, M. A. (2003). Grapevine viroids. In A. Hadidi, R. Flores, J. W. Randles, & J. S. Semancik (Eds.), *viroids* (pp. 195–206). Collingwood: CSIRO Publishing.

Martinelli, L., & Gribaudo, I. (2009). Strategies for effective somatic embryogenesis in grapevine (*Vitis* spp.). An appraisal. In K. Roubelakis-Angelakis (Ed.), *Grapevine Molecular Physiology & Biotechnology, 2nd* (pp. 461–493). Dordrecht: Springer.

Mayer, K. F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., & Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell, 95*, 805–815.

Mica, E., Piccolo, V., Delledonne, M., Ferrarini, A., Pezzotti, M., Casati, C., et al. (2009). High throughput approaches reveal splicing of primary microRNA transcripts and tissue specific expression of mature microRNAs in *Vitis vinifera*. *BMC Genomics, 10*, 558. doi:10.1186/1471-2164-10-558.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473–497.

Navarro, B., Pantaleo, V., Gisel, A., Moxon, S., Dalmay, T., Bisztray, G., et al. (2009). Deep sequencing of viroid-derived small RNAs from grapevine provides new insights on the role of RNA silencing in plant-viroid interaction. *PLoS One, 4*, e7686. doi:10.1371/journal.pone.0007686.

Pantaleo, V., Szittya, G., Moxon, S., Miozzi, L., Moulton, V., Dalmay, T., et al. (2010). Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant Journal, 62*, 960–976.

Polivka, H., Staub, U., & Gross, H. J. (1996). Variation of viroid profiles in individual grapevine plants: novel *grapevine yellow speckle viroid* 1 mutants show alterations of hairpin I. *Journal of General Virology, 77*, 155–161.

Qi, Y., & Ding, B. (2003). Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. *Plant Cell, 15*, 1360–1374.

Rezaian, M. A. (1990). Australian grapevine viroid: Evidence for extensive recombination between viroids. *Nucleic Acids Research, 18*, 1813–1818.

Sano, T., Ohshima, K., Uyeda, I., Shikata, E., Meshi, T., & Okada, Y. (1985). Nucleotide sequence of grapevine viroid: A grapevine isolate of hopstunt viroid. *Proceedings of the Japan Academy, 61B*, 265–268.

Szychowski, J. A., McKenry, M. V., Walker, M. A., Wolpert, J. A., Credi, R., & Semancik, J. S. (1995). The vein-banding disease syndrome: a synergistic reaction between grapevine viroids and fanleaf virus. *Vitis, 34*, 229–232.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., et al. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS One, 2*, e1326. doi:10.1371/journal.pone.0001326.

Wan Chow Wah, Y. F., & Symons, R. H. (1997). A high sensitivity RT-PCR assay for the diagnosis of grapevine viroids in field and tissue culture samples. *Journal of Virological Methods, 63*, 57–69.

Zhu, Y., Green, L., Woo, Y. M., Owens, R., & Ding, B. (2001). Cellular basis of *potato spindle tuber viroid* systemic movement. *Virology, 279*, 69–77.