Viroids in Grapevines: Transmission via Seeds and Persistence in Meristem-regenerated Vines

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at the Department of Plant Science,
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by

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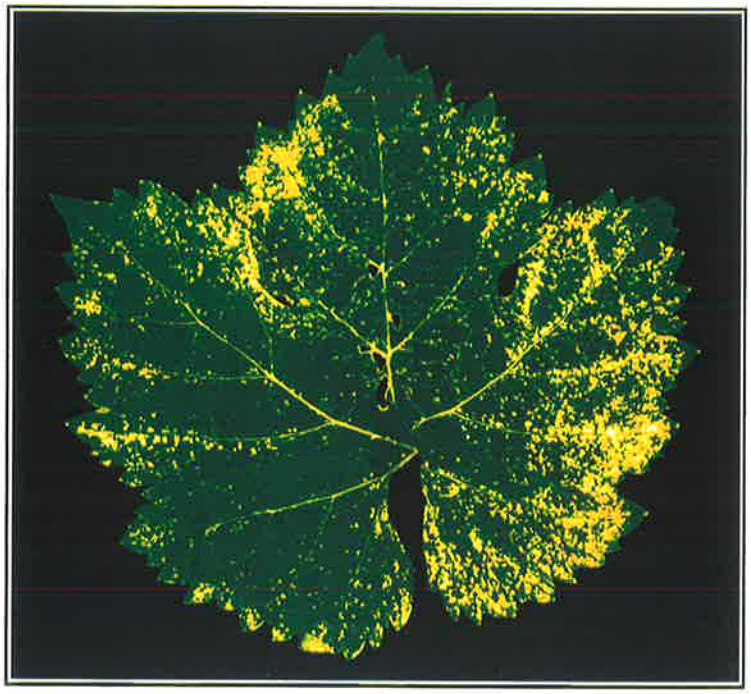
Grapevine yellow speckle symptoms on Chardonnay



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SUMMARY

This general aim of the work described in this thesis was to study viroids in grapevines, especially their vertical transmission via seeds, during meristem culture and micropropagation, and to produce viroid-free vines by shoot apical meristem culture (SAMC). Because of the widespread occurrence of viroids in grapevines, major emphasis has generally been placed on the production of viroid-free vines to understand the true biological significance of viroids in grapevines.

The five viroids in grapevines are the grapevine yellow speckle viroids 1 and 2 (GYSV1 and GYSV2), which are highly specific to grapevines, the hop stunt viroid (HSV), the citrus exocortis viroid (CEV) and the Australian grapevine viroid (AGV), all of which are present in vines with no apparent deleterious effects, except for GYSV1 and GYSV2 which cause the grapevine yellow speckle disease in hot weather.

A prerequisite for this study was a sensitive means of viroid detection. In the first part of this research, an efficient RNA extraction protocol suitable for RT-PCR analysis for all five viroids was developed. The high quality of the RNA extract enabled the development of a highly sensitive RT-PCR assay for the diagnosis of grapevine viroids, significantly more sensitive than other viroid detection techniques currently in use. Careful selection of primers and optimization of the conditions for amplification by fast cycle times was essential for the detection of all five viroids. The amplification of viroids was found to be highly sensitive to the position of PCR primers in relation to viroid structure and the PCR product length. Amplification by the optimized RT-PCR protocol developed here was found to be 25,000-fold more sensitive than dot-blot hybridization analysis using ^{32}P-labelled riboprobes, for a high GYSV1 titre Sultana H5 vine. However, sequence variation was observed to influence the sensitivity of detection of GYSV1 by RT-PCR.

Applications of this RT-PCR-based viroid detection technique included assays for the five viroids in 10 grapevine varieties and for GYSV1 and HSV in SAMC-derived vines and in seedlings. Dot-blot hybridization analysis using ^{32}P-labelled riboprobes

was carried out for GYSV1, HSV, AGV and CEV to confirm the results for viroids present in higher titre. GYSV2 was not assayed separately because it had been reported to cross-hybridise with GYSV1.

Dot blot hybridization assays detected GYSV1, HSV and AGV in the 10 grapevine varieties tested. CEV was not detected. In contrast, the RT-PCR assay detected GYSV1, GYSV2, HSV, AGV and CEV in the 10 grapevine varieties including CEV which previously could not be detected directly by others in vine tissue and required passage through alternative hosts to build up the CEV titre.

Sixty four vines regenerated by SAMC, derived from 14 wine grape varieties and nine rootstocks, were obtained from two separate sources in Australia and assessed for their viroid content. Dot blot hybridization assay showed the persistence at low level of GYSV1 and AGV in almost all the vines tested. HSV was reduced to a level detectable, in most cases, only by RT-PCR. The results obtained from the vines from both sources provided strong evidence that viroids were not eliminated in grapevines by SAMC as reported by other workers, but rather, viroids were differentially reduced. A similar effect was observed in 19 vines regenerated by fragmented shoot apex culture (FSAC).

The indexing of viroids in 11 seedlings demonstrated a viroid content for seedlings, with only GYSV1 and AGV detected in most cases by dot blot hybridization assay, and indicated a differential transmission of viroids via seeds. The detection of viroids in seedlings by RT-PCR was achieved only with the use of an amplification protocol in which the sensitivity was further increased by "Hot Start" PCR. The RT-PCR results confirmed the transmission of GYSV1 in all 11 seedlings and revealed the presence of HSV in 10 seedlings.

A study of the effect of micropropagation on viroid content in grapevine samples generally showed an increase in viroid titre in micropropagated vines compared with their respective stock vines.

The newly-developed RT-PCR assay for viroids was furthermore used in conjunction with SAMC in an attempt to produce viroid-free vines. Stock vines, specially selected for their low viroid titre, were treated in low light and low temperature

this does not produce viroid-free vines
it can only assay for presence of viroids

conditions for up to 7.5 months prior to meristem culture to favour viroid elimination. However, GYSV1 and HSV were detected by RT-PCR in most of the subsequently regenerated vines.

The detection of viroids in grapevines using two different assay techniques highlighted the critical influence of the sensitivity of diagnostic methods. The data obtained so far provided ample evidence that viroids were not easily eliminated from grapevines. The high sensitivity RT-PCR assay was extended to the detection of grapevine leafroll-associated virus-3 (GLRaV-3) and the putative GLRaV-1 in infected grapevines.

STATEMENT

This thesis contains no material which has previously been submitted for an academic record at this or any other university, and is the original work of the author except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

Yan Fong Wan Chow Wah

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Chapter One

Literature Review

1.1. Introduction

Viroids are the smallest plant pathogens known, with single-stranded, circular, covalently-closed, autonomously replicating RNAs as genomes. Viroid genomes are non-encapsidated molecules which range in size from 246 to 375 nucleotides with extensive internal base-pairing resulting in rod-like secondary structures (Sänger et al., 1976).

Viroids are not known to code for any proteins, hence they have to depend on host activities for their replication and biological functions (Sänger, 1987). Since the discovery of the first viroid by Diener (1971), 21 other viroid species have been characterised. A list of viroids, their abbreviations, classification into groups and subgroups and first sequences reported is given in Table 1.1. These data will be referred to throughout this thesis.

Viroids have been found to be the causal agents of economically important plant diseases in spite of their extreme simplicity. From an agricultural crop point of view, viroid-induced symptoms cover the whole range from the slowly developing lethal disease in coconut palms caused by coconut cadang cadang viroid (CCCV) (Hanold and Randles, 1991b) to the symptomless infection of hops with hop latent viroid (HLV) (Puchta et al., 1988). Some host plants appear to recover from viroid infection, but in fact become symptomless while maintaining a stable viroid population, e.g., the avocado sunblotch viroid (ASBV) in avocado trees (Semancik and Szychowski, 1994).

1.2. Biological aspects of viroids

1.2.1. Host range

Viroids have a highly variable host range, the basis of which is unknown. Certain viroids like the potato spindle tuber viroid (PSTV) and the citrus exocortis viroid (CEV) have a broad host range (Diener, 1991); others such as the grapevine yellow speckle viroids 1 and 2 (GYSV1 and GYSV2) are highly specific to *Vitis vinifera* (Taylor et al., 1971, Koltunow et al., 1989). Most viroids are known to have

Table 1.1: Classification of viroids

Viroid-group	Viroid-subgroup	Viroid	Abbrev-iation	Length (nt)	References to sequences
A. ASBV	**ASBV**	Avocado sunblotch viroid	ASBV	246 - 250	Symons, 1981
		Peach latent mosaic viroid	PLMV	337	Hernandez and Flores, 1992b
B. PSTV	**B1. PSTV**	Chrysanthemum stunt viroid	CSV	354, 356	Haseloff and Symons, 1981
		Citrus exocortis viroid	CEV	370 - 375	Visvader et al., 1982
		Citrus viroid IV	CV-IV	284	Puchta et al., 1991
		Coconut cadang cadang viroid	CCCV	246, 247	Haseloff et al., 1982
		Coconut tinangaja viroid	CTiV	254	Keese et al., 1988b
		Columnea latent viroid	CLV	370	Hammond et al., 1989
		Hop latent viroid	HLV	256	Puchta et al., 1988
		Hop stunt viroid	HSV	297 - 303	Ohno et al., 1983
		Potato spindle tuber viroid	PSTV	359	Gross et al., 1978
		Tomato apical stunt viroid	TASV	360	Kiefer et al., 1983
		Tomato planta macho viroid	TPMV	360	Kiefer et al., 1983
	B2. ASSV	Apple scar skin viroid	ASSV	330	Hashimoto and Koganezawa, 1987
		Australian grapevine viroid	AGV	369	Rezaian, 1990
		Citrus bent leaf viroid	CBLV	318	Ashulin et al., 1991
		Citrus viroid III	CV-III	280 -297	Rakowski et al., 1994
		Grapevine yellow speckle viroid-1	GYSV-1	367	Koltunow and Rezaian, 1988
		Grapevine yellow speckle viroid-2	GYSV-2	363	Koltunow et al., 1989
		Pear blister canker viroid	PBCV	315	Hernandez et al., 1992a
	B3. CbV	Coleus blumei viroid	CbV	248	Spieker et al., 1990
		Coleus yellow viroid	CYV	248	Fonseca et al., 1994

dicotyledonous host plants. CCCV and coconut tinangaja viroid (CTiV) are the only two viroids known to infect monocotyledons (Boccardo et al., 1981; Keese et al., 1987). Certain viroids, while causing disease symptoms in one host, replicate in another host without any apparent deleterious effect. Thus while the hop stunt viroid (HSV) causes an economically important disease in hops (Ohno et al., 1983), it is present in grapevines and citrus in a latent state (Sano et al., 1985; Puchta et al., 1989).

1.2.2. Macroscopic disease symptoms

Viroid-induced diseases do not differ significantly from viral diseases in the symptoms they produce. Symptoms at the macroscopic level include various growth abnormalities (epinasty, severe stunting, leaf, tuber and fruit deformities), disturbances of chlorophyll synthesis (chlorotic spots and mosaics), cell death (leaf and veinal necrosis) and the death of whole plants (Matthews, 1992). Cross-protection, a phenomenon frequently observed in virus infections, has also been reported in viroid-infected plants (Niblett et al., 1978). Similarities between symptoms induced by viroids and viruses caused a number of viroid-induced plant diseases to be originally attributed to viruses (for review, see Diener, 1979). This suggests that viroids and viruses probably affect the same or similar plant metabolic pathways.

However, unlike viruses, all viroids are known to infect their hosts in a persistent manner with no cases of recovery. Viroids cannot be eliminated by heat therapy (Taylor and Woodham, 1972) or fragmented shoot apex culture (FSAC) which is known to eliminate viruses from infected plants (Barlass et al., 1982). However, shoot apical meristem culture (SAMC) has been reported to eliminate both viruses and viroids from infected plants (Galzy, 1972; Lizárraga et al., 1980).

1.2.3. Cytopathic effects

Electron microscopical studies have revealed aberrations in the intracellular membrane structures of viroid-infected leaves and membrane-associated

plasmalemmasomes (Semancik and Vanderwoude, 1976), pronounced cell wall distortions and disorganised chloroplasts (Momma and Takahashi, 1983; Bonfiglioli et al., 1994). Metabolic dysfunction at the growing apex of infected plants results in the abnormal formation of cellular membranes in meristematic cells and initiates the development of malformed leaves and stunted growth (Momma and Takahashi, 1983). A change in the sugar and amino acid composition of isolated cell walls from viroid infected cells as compared to non-infected cells has also been reported (Wang et al., 1986).

1.2.4. Localization of viroids in plants

There has been increasing interest recently in the localization of viroids in different plant tissue types as part of an integrated approach to the characterization of the replication strategies and mechanism of pathogenesis. The determination of their cellular localization may be critical to a better understanding of their *in vivo* functions.

The localization of viroids at the histological level using *in situ* hybridization techniques in conjunction with confocal laser scanning microscopy (CLSM) have shown CEV to be localised only in phloem cells of vascular tissues and leaf mesophyll cells while CCCV was found in phloem cells and epidermal cells (Bonfiglioli et al., 1994, 1996). HSV was found in a wide range of cell types apart from the phloem cells, perhaps reflecting an increased mobility for HSV (D. Webb, personal communication).

The ultrastructural localization of viroids by transmission electron microscopy (TEM) has shown ASBV to be located in chloroplasts, mostly on the thylakoid membranes of infected avocado leaf cells (Bonfiglioli et al., 1994), consistent with an earlier fractionation study associating ASBV with the chloroplast fraction (Mohamed and Thomas, 1980). In contrast, the three members of the PSTV group studied so far appear to be in the nucleus, with the subnuclear location differing in each case. PSTV was found in the nucleolus of infected tomato cells (Harders et al., 1989), CEV was evenly distributed across the entire nucleus while CCCV was located in both the nucleoplasm

and the nucleolus of infected oil palm leaves (*Elaeis guineensis*), mostly in the latter organelle (Bonfiglioli et al., 1996).

1.2.5. Ecology and epidemiology

Viroids replicate best at high temperatures (30-33°C) which, together with high light intensity, are known to stimulate rapid symptom expression (Sänger and Ramm, 1974).

Viroids are spread through crops via vegetative propagation, mechanical transmission and through seed and pollen (Matthews, 1992), with the importance of each means varying with the specific viroid and its host. Thus vegetative transmission is dominant for the PSTV and the grapevine viroids, activated in the latter case by the world-wide exchange of grapevine material. Mechanical transmission via farm tools has a significant importance for CEV in citrus and HSV in hops. Seed and pollen transmission have been implicated in the natural spread of ASBV in avocados. Evidence for transmission by aphid species exists for both the tomato planta macho viroid (TPMV) and the tomato apical stunt viroid (TASV) (Diener, 1991).

The ease of transmission of viroids has been ascribed to the secondary structure of the viroids and its complexing to host components during the transmission process. Systemic viroid infection occurs most probably through cell-to-cell movement via plasmodesmata for short distances and via a passive movement mechanism through the phloem for long distance spread (Matthews, 1992; Gafny et al., 1995).

Whilst early work suggested that most viroids are not seed-transmitted, the recent availability of more sensitive detection techniques has resulted in an increasing number of viroids being shown to be vertically transmitted through the pollen and seeds of infected plants (Kryczyñski et al., 1988; Fernow et al., 1970).

1.3. Classification of viroids

Viroids are currently classified into two main groups (Table 1.1) based on comparative sequence analysis (Keese and Symons, 1985; Koltunow and Rezaian, 1989b). The two members of the ASBV group, ASBV and peach latent mosaic viroid (PLMV), are distinguished mainly by the ability of their plus and minus RNAs, the infectious viroid and the viroid complement respectively, to undergo self-cleavage via the hammerhead mechanism (Hutchins et al., 1986). In addition, their sequence and structural features differ from those of other viroids.

All the other viroids belong to the PSTV group which is further divided into three different sub-groups on the basis of three different sets of conserved sequences in the central conserved domain. These are the PSTV sub-group, the apple scar skin viroid (ASSV) sub-group and the coleus blumei viroid (CbV) sub-group.

1.4. Structural features of the viroid genome

Viroid genomes are circular molecules containing intramolecular base-paired regions separated by single-stranded regions forming internal loops to give a linear rod-like secondary structure. Most viroids have been found to exhibit a structural periodicity in their primary structure (Juhasz et al., 1988) with a characteristic repeat unit of 11 or 12 nucleotides for the PSTV subgroup, 60 nucleotides for the ASSV and 80 for ASBV. This periodicity, which may indicate protein-binding ability, has been found in all viroids except HSV.

Viroids also contain prominent polypurine and polypyrimidine tracts which make up a substantial part of their genome (Branch et al., 1993). One of these polypurine tracts is a characteristic feature of the upper strand of the P domain of the PSTV group of viroids (Keese et al, 1988a). Of the other members of the circular subviral RNA pathogens, virusoids, plant viral satellites and the human delta hepatitis agent, only the latter has been found to have this characteristic pattern of nucleotide usage in spite of the widely different host species. Also, viroids and the delta agent do not rely on helper

viruses for their replication like virusoids and plant viral satellites; hence, these tracts may possibly be related to their replication strategy.

Elements of tertiary structure, as shown by the UV-induced cross-linking of G98 and U260 in the highly conserved region of the C domain of PSTV (Branch et al., 1985), may also play a functional role during replication and at the initiation of pathogenesis. The hammerhead structure (See 1.5) which is implicated in the *in vivo* processing of ASBV provides such an example.

1.4.1. Arrangement of the viroid structure into domains

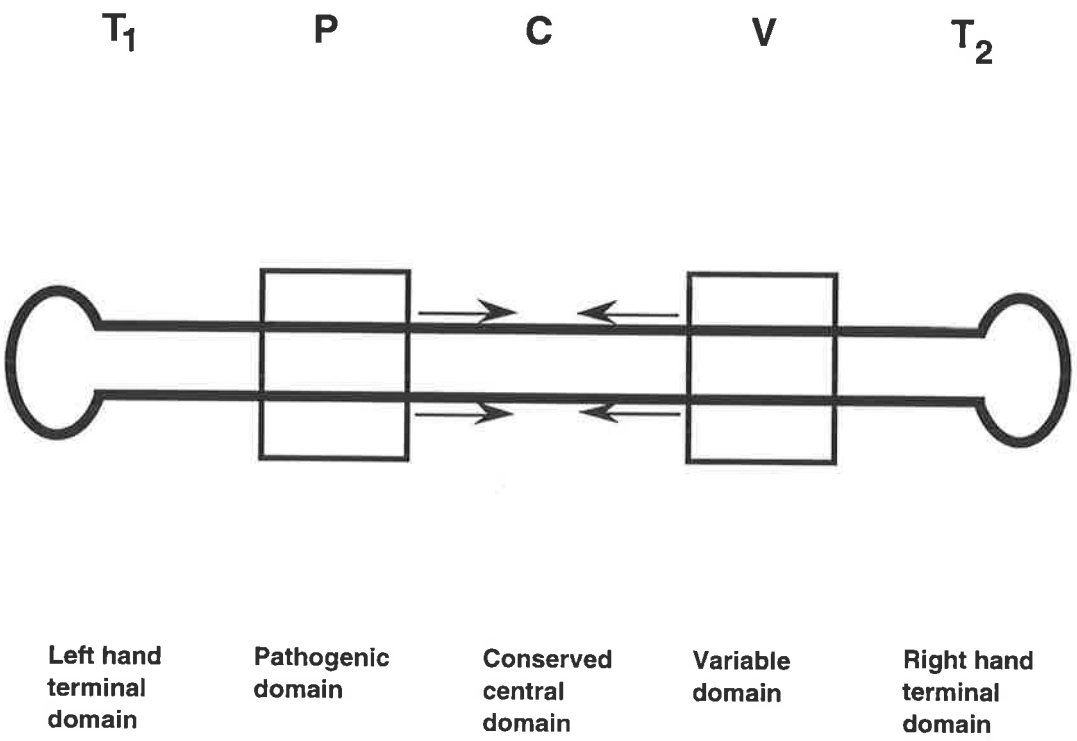
Comparative pairwise sequence analysis of viroids in the PSTV subgroup B1 (Table 1.1) has allowed the arrangement of viroid structure into domains, a model applicable to all viroids except the two members of the ASBV group (Keese and Symons, 1985; Keese et al., 1988a).

Five viroid domains have been defined in the structure of PSTV group of viroids, with the boundaries between domains determined by the marked change in sequence homology on pairwise comparisons between viroids in each subgroup (Fig. 1.1):

(1) the C domain, the most conserved of all domains, which contains a central conserved region (CCR) with sequences of about 30 nucleotides which are highly conserved among the members within each PSTV subgroup, different from those of the other subgroups. The C domain is defined by the ends of a number of inverted repeat sequences in the top and bottom strands. These sequences have the potential to form a conserved hairpin stem. No such common structural feature is found in any of the other four domains. The identity of the repeat sequences determines the further subdivision into three subgroups. The importance of the CCR to the infectivity of viroid cDNA of the PSTV group indicates the CCR as a putative cleavage and ligation site (Visvader et al., 1985; Candresse et al., 1990a).

(2) the pathogenic domain (P) which together with the variable domain (V) shows the most sequence variation within sequence variants (See 1.4.2) of the same viroid

Fig. 1.1: Model of the five viroid domains in the PSTV group of viroids (Keese and Symons, 1985)



The arrows indicate the sites of inverted repeat sequences which can potentially form hairpin stems.

(Visvader and Symons, 1983) and between closely related viroids. The P domain has been associated with symptom expression (Schnölzer et al., 1985; Visvader and Symons, 1986). A common feature in all viroids except CCCV is a long adenine-dominated oligopurine tract, 15-20 nucleotides long on the upper strand.

(3) the V domain, the smallest and most variable of all domains, is also involved in pathogenesis and may determine the level of viroids in infected plants (Visvader and Symons, 1986).

(4) T_1, the left-hand terminal domain, which is highly-conserved within sequence variants of the same viroid. T_1 has been implicated in viroid pathogenesis (Diener, 1993), influencing both the viroid titre and the symptom severity in infected plants. It has been found to be the primary determinant in veinal necrosis.

(5) T_2, the right hand terminal domain, which is also highly conserved, has been implicated in viroid replication. The existence of naturally occurring CCCV and CEV viroids with terminal repeats suggests that T_2 may be a preferred site for RNA recombination (Semancik et al., 1994).

1.4.2. Recombination between viroids

The domain model led to the proposal that the evolution of viroids could possibly occur by the rearrangement of domains as a result of recombination between co-infecting viroids (Keese and Symons, 1985). Indirect evidence in support of this has been provided by the sequences of the columnea latent viroid (CLV) (Hammond et al., 1989) and the Australian grapevine viroid (AGV) (Rezaian, 1990). The nucleotide sequence of CLV shows high sequence homology of its T_1 and T_2 domains to the same domains of PSTV and TASV respectively and contains sequences of TPMV in its P domain and of PSTV and HSV in its C domain. The sequence of AGV shows this viroid to be derived almost entirely from segments of GYSV1, GYSV2, HSV, CEV and ASSV.

1.4.3. Definitions of terms related to sequence variation in viroids

Given the large number of naturally occurring sequence variants that exist in any viroid population, and the correlation of sequence variation to structure and function,the definitions of four terms commonly used in relation to the variation in viroid sequence are given below (Keese et al., 1988a, McInnes and Symons, 1991):

Sequence homology- refers to the percent sequence homology between two viroids as determined by the computer method of Wilbur and Lipman (1983), using defined parameters.

Viroid isolate- refers to the total viroid complement found in a single infected plant. Thus an isolate may contain one or more viroid species, with one or more sequence variants of each viroid species.

Viroid species- refers to one or more independently replicating sequence variants which show more than 90% sequence homology by pairwise comparison. All members have less than 80% - 90% sequence homology with members of any other viroid species.

Sequence variant- refers to an individual viroid molecule of defined sequence. A viroid species contains one or more naturally occurring sequence variants, each of which varies by one or more nucleotides from other sequence variants, but all show more than 90% sequence homology by pairwise comparison. Thus an arbitrary level of 90% sequence similarity currently separates variants from species.

1.5. Replication of viroids

1.5.1. The rolling circle model for viroid replication

Viroids are generally known to replicate via a rolling circle mechanism (Branch and Robertson, 1984; Symons, 1989) resulting in the presence of replicative intermediates, with longer than unit length viroids of plus and minus polarity, in infected cells (Hutchins et al., 1985). According to the rolling circle model of replication, the

circular viroid RNA is transcribed by an unidentified host RNA polymerase into an oligomeric minus strand, which is then cleaved at specific sites to give linear minus strand monomers. After circularization by a host RNA ligase, these minus strands are used as templates for the synthesis of plus strand oligomers according to the same rolling circle mechanism as above and specifically cleaved and ligated to give circular monomeric progeny viroid which accumulate *in vivo*. ASBV has been found most likely to replicate by this pathway (Hutchins et al., 1986).

In a variation of the above mechanism, the multimeric linear minus strand is not cleaved but directly copied into a multimeric linear plus strand which is eventually processed into circular progeny. Most viroids are likely to replicate by this second pathway (Hutchins et al., 1985; Branch et al., 1988).

Although the actual host RNA polymerase involved in viroid replication has not yet been identified, the sites of accumulation of viroid RNA in the cell (See 1.2.4) indicate that RNA polymerase II may be involved in the replication of PSTV (Schindler and Muhlbach, 1992) and CEV (Rivera-Bustamante and Semancik, 1989) and RNA polymerase I in the replication of CCCV (Bonfiglioli et al., 1996). Inhibition of viroid replication by α-amanitine also indicates the involvement of DNA-dependent RNA polymerase II.

1.5.2. Processing of replication intermediates into viroids

1.5.2.1. RNA self-cleavage via the hammerhead structure

Two different mechanisms of processing seem to operate during viroid replication for the specific cleavage of viroid monomers from the concatemeric RNA intermediates. The two viroids belonging to the ASBV group, ASBV and PLMV, are most likely undergo self-cleavage *in vivo* (Hutchins et al., 1986; Hernandez et al., 1992b), through the formation of a characteristic hammerhead structure around the cleavage site formed by the base-pairing of a highly conserved series of short nucleotide sequences (Hutchins et al.,1986; Forster and Symons, 1987a, b; Forster et al., 1987). The hammerhead self-

cleavage reaction, which has been extensively characterised *in vitro* (Sheldon and Symons, 1989), occurs in the complete absence of protein and requires only magnesium ions. The processing of four virusoids has also been found to occur *in vivo* by this mechanism (Forster and Symons, 1987a, b; Davies et al., 1990). RNA self-cleavage via the hammerhead structure has been extensively reviewed in Sheldon et al. (1990) and Symons (1990).

1.5.2.2. Processing of the PSTV group of viroids

Viroids of the PSTV group cannot assume a hammerhead structure. Their mechanism of processing is still poorly understood and remains to be resolved. Evidence suggesting the involvement of a specific endoribonuclease (Tabler et al., 1992, Tsagris et al., 1987) in the production of circular monomers from oligomeric PSTV has been found. Site-directed mutagenesis has defined a potential site for the processing of CEV in the central conserved region (Visvader and Symons, 1986). However, the possibility of self cleavage still remains open for the PSTV group of viroids (Symons, 1991).

1.6. Molecular basis for pathogenesis

Although the molecular structure of viroids has been largely elucidated, the mechanism of viroid pathogenesis is still unknown. The absence of mRNA activity in viroids, as indicated by the lack of conserved open reading frames, implies that viroids exert their pathogenic effects through sequence and structural signals alone and through direct interaction with host constituents. The structural periodicity which has been found only in viroids (See 1.4) and not in other small RNAs provides evidence in favour of their protein binding ability. Hence, approaches towards the elucidation of the molecular basis of disease induction have centered on searches for host constituents with affinities for viroids and sequence homologies with cellular RNAs. Any model for viroid pathogenesis would have to account for the differential response of different host systems to infection by viroid strains of different pathogenicity.

1.6.1. Viroid domains related to pathogenicity

Three of the five viroid domains, P, V and T_1, have been implicated in viroid pathogenesis. The role of the different domains in pathogenicity was elucidated by the sequencing of the full length cDNA clones prepared from sequence variants present in naturally occurring isolates of viroids and the symptom expression obtained on the reinoculation of their RNA transcripts into susceptible plants (Visvader and Symons, 1985; Rakowski and Symons, 1989). Sequence analysis of PSTV, CEV and HSV variants (Schnölzer et al., 1985; Visvader and Symons, 1985; Shikata, 1990) localised the sequence differences in all three viroids within the P and the V domains, indicating the involvement of these two domains in pathogenesis. The transition from a mild to severe symptom expression could be caused by as few as three nucleotide changes (Gross et al., 1981; Visvader and Symons, 1985)

Infectivity studies using chimeric constructs generated *in vitro* by exchanging domains between sequence variants of CEV causing mild and severe symptoms (Visvader and Symons, 1985) indicated that the P domain was primarily responsible for modulating the severity of symptoms and the V domain for influencing the efficiency of infection (Visvader and Symons, 1986). Interspecific chimeric constructs using CEV with mild symptoms and TASV with severe symptoms (Sano et al., 1992), confirmed the above observations and further revealed the involvement of a third domain,T_1, in pathogenicity. The T_1 domain was found to be the primary determinant in the induction of veinal necrosis. The studies also showed that the severity of symptom expression, although influenced mainly by the viroid titre, was also a result of the interactions among the three domains implicated.

1.6.2. Viroid-host constituents

Evidence from Hiddinga et al. (1988) and Diener et al. (1993) indicates that PSTV may induce the phosphorylation of a 68-kD host protein in infected cells, similar to that produced by several viruses. This protein, which is homologous with mammalian P68

protein kinase, is known to be induced by interferon and activated by double-stranded RNA. Once activated, it triggers a series of reactions that culminate in the impediment of protein synthesis initiation (Hershey, 1991). The increase in phosphorylation of the P68 kinase has been found to increase with the level of pathogenicity in PSTV-infected cells (Diener et al., 1993), suggesting the activation of P68 kinase as the triggering event in viroid pathogenesis. An unidentified 43-kD host protein in the nucleus has also been found to bind to PSTV *in vitro* and *in vivo* (Klaff et al., 1989).

1.6.3. Sequence homologies of viroid RNAs with cellular RNAs

Comparison of viroid nucleotide sequences with cellular RNAs has shown putative conserved elements of group I introns in viroids (Dinter-Gottlieb, 1986). However, the absence of these sequences in the hammerhead structure (Diener, 1989) makes their involvement in RNA processing most unlikely. The complementarity between a portion of the negative strand of PSTV and the 5' terminus of mammalian U1 snRNA (Diener, 1981) and the sequence homology of the P domain of PSTV with the 7S RNA from tomato leaf tissue (Haas et al., 1988), have been interpreted as evidence that viroids could possibly exert their effects by interfering with pre-rRNA processing or with the formation of signal recognition particles. However, for such interactions to be part of the mechanism of pathogenesis, they have to explain the varying responses observed in closely-related plant species or cultivars of the same species to infection by the same viroid species and the existence of symptomless plants apparently recovering from viroid infection (See 1.1).

1.7. Viroids in grapevines (*Vitis vinifera* L.)

Five distinct species of viroids, belonging to two different PSTV subgroups, have been confirmed in grapevines and fully characterized by nucleotide sequencing (Rezaian, 1990). They are listed with their characteristics in Table 1.2.

Table 1.2: The five viroids in grapevines and their main characteristics

Viroids	nt	Subgroup	Pathogenicity	Herbaceous host
CEV	371	PSTV	No report	tomato
HSV	297	PSTV	No report	cucumber
GYSV1	367	ASSV	yellow speckle	none
GYSV2	363	ASSV	yellow speckle	none
AGV	369	ASSV	No report	cucumber

Reference:- (Rezaian et al., 1992)
nt: nucleotides

The presence of HSV, the first viroid found in grapevines, was reported by Sano et al. (1985), followed by numerous reports of the widespread occurrence of viroid RNAs in grapevines in Japan, USA, Australia, Europe, Mediterranean and Middle East countries (Flores et al., 1985; Semancik et al., 1987; Rezaian et al., 1988; Minafra et al., 1990). Four other viroids have been reported in grapevines: the CEV, closely related to severe strains of CEV found in tomatoes (Garcia-Arenal et al., 1987), the two closely-related yellow speckle viroids GYSV1 and GYSV2, independently causing the grapevine yellow speckle disease (Koltunow and Rezaian, 1988, 1989a; Koltunow et al., 1989) and the Australian grapevine viroid (AGV) which is considered to be a product of extensive RNA recombination (See 1.4.2) as a consequence of multiple species of co-infecting viroids in grapevines.

Grapevine viroids have been grouped into two classes, according to their titre (Semancik and Szychowski, 1990): (1) high titre-viroids, including HSV, GYSV1 and GYSV2, which can be isolated directly from grapevines and (2) low titre-viroids, including CEV and AGV, which require passage in an alternate host before detection and isolation.

1.7.1. Widespread occurrence of viroids in grapevines

Although grapevine viroids share typical physical characteristics with other viroids, they have an unusual relationship with their hosts in that virtually all grapevines are viroid-infected. The widespread occurrence and persistence of viroids in grapevines possibly occurred through propagation practices and extensive worldwide exchange of grapevine material, providing ideal conditions for the most complete contamination of any single plant group by viroids (Semancik et al., 1992). The simultaneous discovery of grapevine viroids in different geographical regions created a situation whereby a large number of viroid names were given to relatively few viroid species (Semancik and Szychowski, 1992). However, the common identity of the viroids was resolved by RT-PCR analysis (Rezaian et al., 1992).

The ubiquitous occurrence of viroids in grapevines has raised intriguing questions concerning the significance of viroids to vine growth and productivity. To date, the role of viroids in grapevines is unknown. There is no report on their influence on vine growth, grape composition and quality except that observed in relation to the grapevine yellow speckle disease (GYS).

1.7.2. Viroids and diseases in grapevines

The GYS has been confirmed as the only viroid-induced disease of grapevines, caused independently by GYSV1 and GYSV2 (Koltunow et al., 1989). However, there is increasing evidence that GYSV1 may be implicated in a synergistic reaction with grapevine fanleaf virus to produce the vein banding syndrome, a devastating grapevine disease which can cause up to 80% loss in yield (Krake and Woodham, 1983; Szychowski et al., 1995). An enhanced titre of GYSV1 has been observed with the increasing intensity of the vein banding symptom, suggesting a possible linkage between some critical viroid concentration and symptom expression.

The other three viroids seem to be perpetuated in their host in an apparently symptomless condition. Although HSV and CEV cause stunting in other hosts (Diener, 1979), this has not been observed in vines. However, subtle modifications of the growth and development of vines may occur on infection that have not been observed until now because of the lack of viroid free control vines. The lack of pathogenicity observed in grapevines, in spite of the multiple viroid infection, is not surprising since obvious detrimental effects would have limited the propagation of infected vines.

1.7.2.1. The grapevine yellow speckle disease (GYS)

GYS was first observed by Taylor and Woodham (1972) on Australian grapevine cultivars as well as on imported Californian vines. Strong symptoms usually develop in midsummer and intensify in the hotter months. Typical symptoms consist of small irregular specks ranging from pinpoint to 1 mm in size, scattered over the leaf surface but

mainly associated with the veins. Speckles appear on a limited number of leaves, usually two to three leaves per vine but rarely more than twenty, changing from yellowish-green to chrome yellow as the disease progresses. GYS, which is widespread in Australia, has been reported to lower yield and berry sugar, with compounded effects when mild strains of leafroll are also present (Nicholas et al., 1994).

Although the two yellow speckle viroids, GYSV1 and GYSV2, have been shown to cause the yellow speckle disease in Australia, vines containing these two viroids have not displayed yellow speckle symptoms to date under Californian field conditions (Szychowski et al., 1995). Cultivars, known to be infected, only express GYS when grown at 32°C under continuous light (Mink and Parsons, 1975).

1.7.2.2. Variability of symptoms of the GYS disease

Symptom expression of the GYS is known to be extremely variable and erratic, related to cultivar, age of plants, environmental and seasonal factors (Shanmuganathan and Fletcher, 1980). The worldwide distribution of viroids in grapevines has raised doubts as to whether viroids are indeed the primary causal agents in the expression of GYS disease. The extreme variability in symptom expression coupled with its erratic occurrence may be caused by a stress condition induced by a hot climate and aggravated by the presence of viroids. Hence viroids may simply be a factor in disease expression, leaving open the possibility of a more complex aetiology for viroid-induced grapevine diseases.

The wide variability in symptom expression may be related to the fact that the two yellow speckle agents, GYSV1 and GYSV2, only share partial (73%) sequence homology (Koltunow et al., 1989). The existence of a large number of GYSV1 sequence variants may be a further contributing factor (Rigden and Rezaian, 1993; Polivka et al., 1996). Most of these variants can give rise to two distinct alternative structures in the P domain (Rigden and Rezaian, 1993) that could possibly contribute to

variable symptom expression through the differential build-up of the two types of sequence variants in vines containing a mixed population.

1.7.3. Spread and transmission of viroids in grapevines

Contradictory evidence exists on the spread of viroids. Although Taylor and Woodham (1971) found no evidence for the natural spread of the yellow speckle disease in the field, Szychowski et al. (1988) reported the spread of grapevine viroids by mechanical inoculation among vines during routine cultural practices. However, recent epidemiological studies carried out on viroid distribution in two vineyards (Staub et al., 1995b) do not support the transmission of viroids by normal pruning. Rather, the data suggest the independent evolution of viroid populations within single vines, based on the different GYSV1 and HSV variant profiles found in neighbouring vines together with the random distribution pattern of the viroid variants through the vineyards.

Szychowski et al. (1988), from a study of viroid profiles in rootstocks in California, suggested the systemic transmission by viroid-infected rootstocks as a possible factor in the widespread distribution of viroids in grapevine. However, the analysis of viroid variant profiles in rootstock clones and different grapevine cultivars in Germany (Staub et al., 1995b) indicate that viroid transmission is more likely to occur through infected scion varieties rather than through infected rootstocks.

No evidence for the transmission of viroids via grapeseeds has been reported so far (Taylor and Woodham, 1972; Semancik et al., 1987; Koltunow and Rezaian, 1988; Koltunow et al., 1988; Minafra et al., 1990).

1.7.4. Viroid profiles in grapevines

Analysis of viroid-infected vines by sequential polyacrylamide gel electrophoresis (sPAGE) (See 1.8.3) has revealed the existence of viroid profiles, based on the detection of viroid bands on the gels after silver staining, at positions corresponding to those of the known grapevine viroids (Semancik et al., 1987). In comparative studies between

Europe, California and Spain (Szychowski et al., 1991; Duran-Vila et al., 1990), three major viroid profiles were reported: (1) HSV only, (2) GYSV1/ HSV and (3) GYSV1/ GYSV2/ HSV. Grapevine profiles were found to differ according to the origin of the vines, with single infections of GYSV1 or HSV relatively rare and GYSV2 associated mainly with the mostly self-rooted table grapes (Duran-Vila et al., 1990). Vines solely infected with GYSV1 and GYSV2 without HSV have never been found (Minafra et al., 1990; Szychowski et al., 1991; Staub et al., 1995b). The presence of HSV in nearly every vine studied was confirmed by studies on Californian and Australian vines (Rezaian et al., 1992).

The viroid profiles, when viewed against the grouping of vines into wine, table or rootstock cultivars, brought out distinct patterns possibly related to cultural practices and usage patterns (Semancik et al., 1992). Thus, the HSV only profile was present evenly in all three groups of cultivars, the GYSV1/HSV profile was predominant in wine grapes and the GYSV1/GYSV2/HSV profile was found in table grapes.

1.7.5. A positive influence for viroids in grapevines?

The significance of the ubiquitous presence of viroids in vines remains a virological enigma. The possibility that viroids could have been maintained in grapevines via the selection mechanism for desirable vine traits has been raised, implying a positive role for viroids in vine growth and viticultural characteristics (Semancik et al., 1987). The existence of variations in viroid profiles, quantitative as well as qualitative in selections within the same grapevine varieties, suggests that the viroid complement in grapevines may have a role in clonal differences. Such a role would not be inconsistent with reports from Cameron in Western Australia (1984) which indicated a substantial improvement of table grape quality of the variety Emperor upon infection by an undefined graft-transmissible factor. Although the original Emperor clone "3A" was known to be infected with a mild leafroll virus strain, the qualities induced by bud inoculation with this clone were inconsistent with those normally associated with leafroll. While leafroll virus

normally causes a lowering of pH and the sugar content in infected grapevines (Walter, 1988), tablegrapes infected by graft-inoculation with Emperor clone "3A" were found to have a higher pH and a higher sugar content, suggesting the involvement of pathogens other than viruses. The presence of grapevine leafroll-associated virus type 5 (GLRaV-5) has now been confirmed in the Emperor 3A clone using the direct double antibody sandwich technique (dDAS-ELISA) with a biotin strepavidin detection system (A. Ewart, personal communication); however, this does not rule out the possible involvement of viroids.

A higher efficiency in biomass accumulation and survival advantages, reported in CEV-infected tomato suspension cell cultures as compared to healthy cells in culture (Duran-Vila et al., 1995), would also be consistent with a positive influence of viroids.

1.7.6. The need for viroid-free grapevines

The lack of viroid-free vines means that every vine response, from viticultural characteristics and disease expression to wine quality has been observed until now through a viroid background (Semancik, 1993). The role of viroids and their effects on vine growth and vine performance remain to this day unknown mainly because of the ubiquitous presence of viroids in vines and the absence of alternate herbaceous hosts for the two yellow speckle disease-causing viroids. The availability of viroid-free grapevines is thus critical to an evaluation of the true biological significance of viroids in vines. Hence major emphasis has been placed on the production of viroid-free vines to resolve this issue.

Seedlings have been used as a source of viroid-free material because numerous reports have indicated that viroids are not present in seedlings (Taylor and Woodham, 1972; Semancik et al., 1987; Koltunow and Rezaian, 1988; Koltunow et al., 1988; Minafra et al., 1990). Viroid-free vines, produced by SAMC were reported by Duran-Vila et al. (1988), thus making available viroid-free clones of known cultivars. Viroid-free vines from some 32 grape varieties, including wine and table grape varieties and

rootstocks were thus produced (Juarez et al., 1990). Field tests involving the re-introduction of viroids into putative viroid-free vines were carried out at the Oakville Experiment station in Napa Valley, California, and have shown no significant differences so far between viroid-free and viroid-reinfected vines (Wolpert et al., 1993, 1996). Both vine seedlings and SAMC-derived vines have been used in re-inoculation studies (Szychowski et al., 1988; Rigden and Rezaian, 1993; Szychowski et al., 1995).

Central to all these experiments is the assumption of the viroid-free status of the vines used for re-inoculation. Reports of meristem culture-regenerated vines and vine seedlings as viroid-free material were based on techniques available at the time, namely bioassays, polyacrylamide gel electrophoresis (PAGE) analyses and Northern blot hybridization assays with cDNA probes (See 1.8). In recent work using more sensitive diagnostic methods, HLV was detected in all progeny hops regenerated from meristems (Morton et al., 1993). Hops found negative by hybridization methods using ^{32}P-labelled riboprobes retested positive by reverse transcription-polymerase chain reaction (RT-PCR) assay (Hataya et al., 1992) (See 1.8.4.2 and 1.8.5). Hence the "viroid-free" status of vines may need to be reconfirmed with more sensitive diagnostic methods before further experimentation.

1.8. Diagnostic procedures for viroids

Diagnostic methods applicable to viroids have been described exhaustively by McInnes and Symons (1991) and have recently been reviewed by Hanold (1993). Techniques for viroid detection have consistently evolved towards simplified procedures with greater sensitivity.

1.8.1. Biological methods of indexing

Diagnostic techniques for viroids have traditionally involved biological methods of indexing involving sap or graft inoculation of infected material to suitable indicator hosts. Although sensitive and indicative of the infectious nature of the ~~host~~ viroid, biological

indexing has proved to be an inadequate means for viroid testing, with symptom expression dependent on the host as well as environmental conditions (Taylor and Woodham, 1972), and usually requires more than one indicator plant over a prolonged incubation period for diagnosis (Cirami et al., 1988). Apart from the high labour costs and the need for additional testing to identify the actual viroid involved, the nature of the testing also precludes the detection of viroids prior to symptom expression. Thus the presence of the two yellow speckle viroids in asymptomatic Californian vines could only be shown by molecular techniques (Rezaian et al., 1992).

1.8.2. Electron microscopy

Until recently, the use of electron microscopic techniques has not been of great relevance to viroid detection compared to virus detection because no characteristic particles could be readily detected. The recent use of confocal laser scanning microscopy in conjunction with *in situ* hybridization and other molecular techniques using specific nucleic acid probes and different fluorescent reporter molecules has allowed the precise localization of viroids at the tissue level (Bonfiglioli et al., 1994, 1996), while ultrastructure studies with transmission electron microscopy have localised PSTV, CCCV and CEV in the nucleus and ASBV in the chloroplasts (See 1.2.4).

1.8.3. Polyacrylamide gel electrophoresis (PAGE)

Viroids generally occur in low concentration in infected plants so that a partial purification and concentration of the nucleic acid extracts is required before analysis in an appropriate PAGE system. Detection of viroids is carried out by the separation of nucleic acids, total or partially fractionated, in an electric field before staining and visualization of the viroid RNA. Among the more widely used techniques are two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (Schumacher et al., 1983), bidirectional gel electrophoresis and return gel electrophoresis (R-PAGE) (Schumacher et al., 1986) and sPAGE (Semancik et al., 1987), followed by the detection of low levels of viroids

by silver staining, which was reported to be 3-100 fold more sensitive than ethidium bromide or toluidine blue (Igloi, 1983).

The presence of multiple viroids in grapevines was first indicated by the observation of several bands in native and denaturing PAGE (Flores et al., 1985) and confirmed by the presence of multiple distinct bands of circular RNA on 2-D gels (Rezaian et al., 1988). The first grapevine viroid profiles (See 1.7.2) were thus derived from the electrophoretic pattern obtained from the viroid RNA extracts on sPAGE (Semancik et al., 1987; Szychowski et al., 1991).

Various procedures have since increased the sensitivity of viroid detection by PAGE. However, these methods are to present-day diagnostic standard overwhelmingly laborious because of the need to extract huge amounts of infected tissue. Earlier workers extracted 500 g batches of leaves (Palukaitis and Symons, 1980), reduced with the refinement of techniques of electrophoresis to lesser quantities, 150 g (Flores et al., 1985) and, more recently 10 g (Duran-Vila et al., 1988). Moreover, they do not have the sensitivity of the more recent techniques.

1.8.4. Nucleic acid hybridization

Nucleic acid hybridization-based techniques have been extensively used for viroid detection because of their high specificity and sensitivity. Earlier indexing involved a liquid hybridization procedure (Palukaitis et al., 1981), reported to have a superiority of at least 1000-fold over polyacrylamide gel electrophoresis for the detection of ASBV. However liquid hybridization techniques are tedious, of variable efficiency and not suited to large scale indexing, and have since been superseded by dot blot hybridization procedures.

1.8.4.1. Dot blot hybridization

Dot blot hybridization, the most widely used procedure for viroid detection, has been over a long period of time the most sensitive, specific, convenient and reliable

diagnostic procedure. The use of highly specific probes allows the identification of most viroids. Dot blot hybridization has been reported to be at least 10-fold more sensitive than PAGE (Owens and Diener, 1981) and 4-6 fold more sensitive than R-PAGE (Singh et al., 1994).

Dot-blot hybridization involves the immobilization of denatured viroid RNA on a support such that the bound sequences can hybridise to an added nucleic acid probe. Detection of the viroid is achieved by using probes labelled either with radioactive isotopes e.g., ^{32}P, or non-radioactive ligands such as biotin (Roy et al., 1989), photobiotin (McInnes et al.,1989) or digoxigenin (DIG) (Welnicki and Hiruki, 1992). Excess non-hybridized probes are removed after hybridization by highly stringent washing. The labelled viroids are then detected by the appropriate means.

Since its first application to PSTV by Owens and Diener (1981), several modifications have been reported to simplify procedures and to improve sensitivity. Innovations introduced to minimise sample manipulations include direct tissue blot (Podleckis et al., 1993; Romero-Durbán et al., 1995) in the place of partially purified nucleic acid dots (Barker et al., 1985; Salazar et al., 1988). However the sensitivity of viroid detection was lower by a factor of 3-10 in direct blotting (Candresse et al.,1990b) because of the inhibitory effect of plant sap on hybridization (Owens and Diener, 1981; Candresse et al., 1988). The direct use of sap also causes a high non-specific background (Macquaire et al., 1984; Flores et al., 1986) especially in grapevines (Romero-Durbán et al., 1995). Hence a reduction in the number of processing steps may be achieved at the expense of accuracy and is not applicable where high sensitivity is the aim.

1.8.4.2. Sensitivity of viroid detection using different probes

The sensitivity of viroid detection by hybridization has been found to vary with the specific host plant and the nature of the probes. Table 1.3 shows the limit of sensitivity of detection reported for PSTV, probably the most well studied viroid, using

different probes and labelling methods. Double-stranded DNA probes, radioactively-labelled either by nick translation (Rigby et al., 1977) or random priming (Feinberg and Vogelstein, 1983), were among the earlier probes made. These were rapidly superseded by full-length single-stranded cDNA (Barker et al., 1985) and cRNA probes (Lakshman et al., 1986), found to be more efficient than double-stranded probes. Of these two types of probes, cRNA was found to be superior to cDNA, with the more stable RNA/RNA duplexes giving a higher sensitivity as well as a lower background. An advantage of 4-32 fold higher sensitivity of cRNA over cDNA has been reported for detection of PSTV in tomato leaf (Lakshman et al., 1986, Candresse et al., 1990b) and 2-30 fold in potato leaf (Singh et al., 1994). The use of multimeric (hexameric) probes has been reported to give a further four fold increase of sensitivity over monomeric probes (Welnicki and Hiruki, 1992; Singh et al., 1994).

Non-radioactive probes were developed for the purpose of routine testing because of the considerable advantages of cost and labour associated with prolonged stability of the probes, reduced health and safety risks as well as a lack of requirement for special precautions, e.g. waste disposal, special training and laboratories. Until recently, non-radioactive probes have generally been considered to have a sensitivity far below that of radioactive probes (Candresse et al., 1990b) (See Table 1.3), although biotinylated probes have been reported to have a sensitivity comparable to that of radioactive probes (Roy et al., 1989; McInnes et al., 1989). However, the combination of DIG-labelled probes with chemiluminescent substrates instead of colorigenic substrates has enabled the reduction of non-specific binding and a sensitivity equal to (Welnicki and Hiruki, 1992; Podleckis et al., 1993; Romero-Durbán et al., 1995) or even higher than that of radioactive probes (Singh et al., 1994, Werner et al., 1995) has been reported.

Thus, with updated probe technology, the sensitivity of viroid detection has improved considerably. To summarize the data in Table 1.3, if a limit detection of 1pg for cRNA probes is used as a basis for the comparison of the sensitivity obtained with different probes, cRNA probes are found to achieve a sensitivity of 5-250 fold higher

Table 1.3: Detection of PSTV by dot blot hybridization

Probe/label	Probe preparation	Sensitivity limit (pg)	References
cDNA / ^{32}P	nick translation	250[a]	Macquaire et al., 1984
cDNA / ^{32}P	nick translation	150[a]	Owens and Diener, 1981
cDNA / ^{32}P	nick translation	100	Salazar et al., 1988
cDNA / ^{32}P	M13 primer extension (-)	100	Salazar et al., 1988
cDNA / ^{32}P	M13 primer extension (+)	10-20	Salazar et al., 1988
cDNA / ^{32}P	nick translation	50[a]	Bernardy et al., 1987
cDNA / ^{32}P	nick translation	11.2[a]	Lakshman et al., 1981
cDNA / ^{32}P	nick translation	5-10	Candresse et al., 1990b
$cDNA^{m}$ / ^{32}P	random primer extension	5	Welnicki and Hiruki., 1992
cRNA / ^{32}P	RNA transcription	1.4[a]	Lakshman et al., 1986
cRNA / ^{32}P	RNA transcription	0.33	Salazar et al., 1988
cRNA / ^{32}P	RNA transcription	2-4	Roy et al., 1989
cRNA / ^{32}P	RNA transcription	1-2	Roy et al., 1989
cRNA / ^{32}P	RNA transcription	>1	Candresse et al., 1990b
cDNA / biotin	nick translation	150	Candresse et al., 1990b
cRNA / biotin	RNA transcription	5	Candresse et al., 1990b
$cDNA^{m}$ / DIG	random primer extension	7.0[c]-3.9[bd]	Singh et al., 1994
$cDNA^{m}$ / DIG	random primer extension	2.5[d]	Welnicki and Hiruki, 1992
$cRNA^{m}$ / DIG	RNA transcription	1.2[c]-0.48[bd]	Singh et al., 1994

a dilution in clarified sap.
b dilution in yeast t-RNA; dilution was in buffer or water in all other cases.
c with colorigenic substrate
d with chemiluminescent substrate
m concatemeric probe X6.2 PSTV monomer.

than that obtained with cDNA probes, all categories included. Non-radioactive multimeric probes are found to achieve a sensitivity comparable to that of radioactive probes.

Although widely used for viroid indexing programs on a large scale, dot-blot hybridization remains an extremely laborious technique with tedious sample preparations and a lag time of several days before a diagnosis can be made. Its major limitations include an inadequate sensitivity for low viroid load samples and cross-hybridization between viroids with high sequence homology (Koltunow et al., 1989).

1.8.5. Reverse transcription and polymerase chain reaction (RT-PCR)

Since the polymerase chain reaction was first developed by Saiki et al. (1985), the versatility of this technique and its profound impact, specially in the diagnostic field, have been widely recognized. RT-PCR involves the synthesis of a cDNA copy of a template RNA and its subsequent amplification by PCR using specific primers. The choice of primers determines the specificity while the sensitivity stems from the exponential amplification of templates. The PCR has been generally acknowledged as a powerful and exquisitely sensitive technique (Mullis and Faloona, 1987) that can dramatically improve the detection of low copy number target molecules not detectable by any of the other techniques described. However, the use of RT-PCR as an indexing technique requires an optimisation of the amplification conditions to minimise the interference with the doubling of the target sequence at each cycle (Ferré, 1992) and to maximise reproducibility between assays.

Advantages of RT-PCR include minimal sample requirement with no need for viroid enrichment, a short time lag for diagnosis and the optional use of radioactivity. The minimal sample requirement for PCR has brought about the development of "fast extraction" methods from milligram quantities of fresh material, significantly simpler than previous extraction protocols and more suitable for large scale indexing (Levy et al., 1994, Steiner et al., 1995, Thomson and Dietzgen, 1995). These methods usually

involve a one or two step extraction process with the supernatant used directly for PCR, thus, saving on time, labour and reagents.

The most significant advantage remains the considerable increase in sensitivity obtainable. A direct comparison of RT-PCR assay to dot-blot hybridization in relation to the detection of viroids has not been found from a search in the literature. A comparison of the sensitivity of detection of plant viruses in different hosts by these two techniques is given in Table 1.4. These few examples provide an interesting insight into the potential of RT-PCR and the practical reasons that may prevent this technique from reaching its potential. A wide range in the increases of sensitivity of RT-PCR over dot blot hybridization (Table 1.4, column 3) is observed, influenced by many factors among which are, the host system, the nature of the hybridization probes, the purity of the templates, the presence of inhibitors to the RT-PCR enzymes and the efficiency of the different amplification protocols. Table 1.4 shows that the sensitivity obtainable by RT-PCR by far surpasses that which can be achieved by even the most up-to-date hybridization procedure. However, while comparisons tend to be made between RT-PCR and dot blot hybridization, a comparison of the sensitivities between the two assay methods reported by different workers shows as wide a gap as that observed between the two different techniques. Thus, a 10^7 fold difference exists between the highest and the lowest increase of sensitivity of RT-PCR over dot blot hybridization reported (Borja and Ponz, 1992; Simes and Symons, unpublished data). However, the figure reported by Borja and Ponz appears unusually high compared to that reported by other workers. Taking into consideration the 10^4 fold increase in sensitivity reported by Hu et al. (1995), a 1000-fold difference in sensitivity between RT-PCR results is still apparent.

RT-PCR is, however, associated with a number of serious drawbacks. One of the common problems encountered stems from its very high sensitivity which can influence the outcome of the results with minimal variations in amplification conditions. False positive results attributable to contamination and false negatives as a result of enzyme inhibition or suboptimal amplification conditions are major problems in

Table 1.4: Sensitivity of virus detection in plants by RT-PCR and dot-blot hybridization assay

Virus	Host	RT-PCR: Dot blot	PCR-Limit sensitivity*	References
GVA closterovirus	grapevine	200: 1[a]	100 pg	Minafra et al., 1992a,b
Potato leafroll virus	potato	10: 1[b]	ns	Simes and Symons, unpublished data
Cucumber mosaic virus	banana	10^4: 1[a]	0.1 pg(purified)[§]	Hu et al., 1995
Cucumber mosaic virus	banana	10^2: 1[a]	10 ng (crude)	Hu et al., 1995
Artichoke mottle crinkle tombusvirus	artichoke	20: 1[c]	5 pg	Barbarossa et al., 1994
Artichoke mottle crinkle tombusvirus	artichoke	1: 2.5[d]	5 pg	Barbarossa et al., 1994
Cherry leafroll virus	tobacco	10^8: 1[a]	5 pg	Borja and Ponz, 1992
Plum pox potyvirus	peach	25: 1[d]	10 fg	Wetzel et al., 1991

*, in total nucleic acid extracts.

§, not specified whether in total nucleic acid extracts or otherwise.

ns, not specified.

Dot blot hybridization probes were in a, ^{32}P-labelled random-primed DNA; b, ^{32}P-labelled nick-translated DNA; c, sandwich hybridization using a biotinylated transcript and a digoxigenin-labelled probe; d, ^{32}P-labelled riboprobe.

diagnostic work (Bockstahler, 1994). Contamination with PCR product "carryover" from previous amplifications in routine indexing work is an equally serious handicap (Kwok and Higuchi, 1989: Yap et al., 1994).

1.9. Aims

The aims of the work that is described in this thesis were:

(1) to develop a simple and efficient protocol for the extraction of viroid RNA from grapevine tissue that is compatible with RT-PCR analysis.

(2) to develop a high sensitivity RT-PCR assay that is reliable for the diagnosis of the five viroids in grapevines.

(3) to apply this RT-PCR assay to investigate:

(a) the elimination of viroids in vines by SAMC.

(b) the transmissibility of viroids via seeds in grapevines.

(4) to produce vines indexed viroid-free by RT-PCR.

Chapter Two

General Materials and Methods

2.1. Materials

All general laboratory reagents were of at least analytical grade in standard. Suppliers of enzymes and chemicals are specified only where alternative sources might affect performance or quality of reagents. Solutions were prepared under sterile conditions with ultra-pure MilliQ water, and autoclaved where appropriate. Alternative sterilization for small volumes and non-autoclavable materials was by filtration through a Millex-GS 0.22 μm filter (Millipore, USA).

2.1.1. Synthetic oligodeoxyribonucleotides

Synthetic oligodeoxyribonucleotides were prepared on a DNA synthesiser (Applied Biosystems, Model 380B, USA) by Dr Neil Shirley of the Nucleic Acid and Protein Chemistry Unit of the Special Research Centre, Department of Plant Science, University of Adelaide. Oligonucleotides were purified by ion exchange HPLC using a MonoQ column (Pharmacia, USA). Sequences of oligonucleotides are given in the text of this thesis.

2.1.2. Nucleotides and radionucleotides

Ultrapure nucleotide triphosphates (NTPs) and deoxynucleotide triphosphates (dNTPs) were obtained from Pharmacia. α-^{32}P-labelled dATP and dCTP (10 mCi/ml) and α-^{32}P-UTP (10 mCi/ml) were obtained from Bresatec (Australia).

2.1.3. DNA molecular weight markers

pUC 19 DNA plasmid digested with *Hpa*II (Bresatec, Australia) was used as low molecular weight markers. Medium range molecular weight markers used were the 1 kb DNA ladder from GibcoBRL (Australia) and SPP-1 DNA digested with *Eco*R 1 (Bresatec, Australia).

2.1.4. Sample loading buffers

3x Urea loading buffer (ULB): 50 mM Tris-HCl, 10mM EDTA, 20% ($^{w}/_{v}$) sucrose, 2 M urea, 0.06% ($^{w}/_{v}$) xylene cyanol, 0.06% ($^{w}/_{v}$) bromophenol blue.

2x Formamide loading buffer (FLB): 95% formamide, 10 mM EDTA pH 8.0, 0.02% ($^{w}/_{v}$) xylene cyanol, 0.02% ($^{w}/_{v}$) bromophenol blue.

2.1.5. Bacterial strains, growth media and cloning vectors

The following *Escherichia coli* K12 strains were used:

(1) *E. coli* DH 5α (*sup*E44 Δ*lac*U169 (Δ80 *lac*ZΔ M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1; BRL, USA) was used for all routine cloning work.

(2) *E. coli* XL1-Blue (*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 *lac*$^{-}$ F'[*pro* AB^{+} *lac* I^{q} *lac*ZΔ M15 Tn10 (*tet*^{r})]; Stratagene, USA) was used as host for the M13 bacteriophage.

E. coli cultures were grown in Luria (LB) broth (1%($^{w}/_{v}$) bacto-tryptone, 0.5% ($^{w}/_{v}$) yeast extract, 1% ($^{w}/_{v}$) NaCl, pH 7.0) or 2YT (1.6% ($^{w}/_{v}$) bacto-tryptone, 1% ($^{w}/_{v}$) yeast extract, 0.5% ($^{w}/_{v}$) NaCl, pH 7.0). Ampicillin, at a concentration of 50-100 μg/ml, was added to the growth media where required.

Bacteria were plated out on LB broth agar plates containing 1.5% ($^{w}/_{v}$) Bacto-agar (Difco, USA). Cloning was carried out using the vectors pBluescript SK^{+} (Stratagene, USA) and pGEM 5Zf^{+} (Promega, USA).

2.2. Molecular biology methods

Methods were performed essentially according to standard procedures described in Sambrook et al. (1989) or using specifications given by the manufacturers except where indicated. Routine procedures used, suppliers and solution components are recorded here. Specific methods used are listed in each chapter.

2.2.1. Restriction digestion of DNA

DNA was digested with restriction endonucleases supplied by Boehringer Mannheim (Germany), Promega (USA), New England Biolabs (USA) or Bresatec

(Australia), using buffer systems recommended or supplied by the manufacturers. Restriction digests using two enzymes were carried out using buffer conditions as close as possible to that recommended for each enzyme alone. In analytical digests, 0.5-1µg of DNA was incubated with 2-5 units each of the appropriate restriction enzymes in a reaction volume of 20 µl for a minimum of 2 h.

2.2.2. Gel electrophoresis

2.2.2.1. Agarose gel electrophoresis

Agarose minigels were prepared from 0.7-2.0% ($^{W}/_{V}$) solutions of agarose from SeaKem GTG:Genetic Technology Grade (FMC, USA) in 1xTBE (89 mM Tris-borate, pH 8.3, 2mM EDTA). Minigels containing 3.0-4.0% ($^{W}/_{V}$) solutions of agarose were prepared from 2.25-2.5% Nusieve, GTG:Genetic Technology Grade™ (FMC, USA) added to 0.75-1.5% SeaKem GTG:Genetic Technology Grade (FMC, USA) were used to separate DNA fragments of less than 120 bp size. Ten ml of the molten agarose was poured on to a 7.5 x 5.0 cm glass plate after positioning of the appropriate gel comb. Samples were mixed with a 0.5 volume of 3xULB (See 2.1.4) before loading into the wells. Gels were electrophoresed in 1xTBE running buffer at 80-120 mA, stained by soaking in ethidium bromide solution (10 µg/ml ($^{W}/_{V}$) in water) for 4 min and destained in water (20-45 min) before visualization under a short wavelength UV transilluminator.

2.2.2.2. Polyacrylamide gel electrophoresis

Polyacrylamide minigels (9x7.5x 0.075 cm) were prepared from 10 ml solutions containing 5-20% ($^{W}/_{V}$) polyacrylamide (Ultrapure Accugel 19:1 sequencing grade) in 1xTBE, 100 µl of freshly prepared 10% ($^{W}/_{V}$) ammonium persulphate and 5 µl TEMED (N, N, N', N'-Tetra-methylethylenediamine). Denaturing gels were prepared by the addition of 7 M urea. Gels were allowed to polymerise for 30 min and then pre-electrophoresed for 20 min at 120 V. Samples (15-20 µg nucleic acids in 5 µl volume) were mixed with an equal volume of 2xFLB (See 2.1.4), denatured by heating for 1 min at 80°C and loaded immediately. Electrophoresis was carried out in a BIO-RAD Mini

Protean Cell II with 1xTBE as running buffer for 1.5 h at 110-120 V and the nucleic acid bands visualised as described in section 2.2.2.1.

2.2.3. Purification of DNA fragments from gels

DNA fragments of interest, after separation by electrophoresis in a 1-2% agarose gel (See 2.2.2.1), were excised from agarose gels after detection with ethidium bromide and visualization under long wavelength UV light. DNA was extracted from the gel slices by the Geneclean procedure, using kits from Bio 101 (USA) or Qiaex II gel extraction kit from Qiagen (Germany).

Fragments, smaller than 100 bp, were separated in a vertical 5-8% polyacrylamide gel, excised from the gel and the DNA eluted from the gel slice by incubation in 400 µl of 0.5 M ammonium acetate, 0.1% SDS, at 37°C for 16 h. The DNA was precipitated by the addition of 2.5 vol of 100% ethanol, washed in 70% ethanol, air dried and resuspended in 10-20 µl of 0.1 mM EDTA.

2.2.4. Purification of nucleic acid samples on Sepharose CL-6B spin columns

Eppendorf tubes (0.5 ml), with the bottom pierced with a 21G needle, were placed in 2 ml microfuge tubes. One drop of acid-washed glass beads (212 - 300 µm, Sigma) was placed in the bottom of each tube which was then filled with Sepharose CL-6B resin (Pharmacia) equilibrated in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The tubes were spun in a bench centrifuge (Eppendorf, Germany) at 1800 rpm for 3 min to remove the excess TE buffer. The 2 ml microfuge tubes were replaced by intact tubes and the nucleic acid samples were loaded on to the top of the column and the spin repeated. The DNA was collected in the intact tubes.

2.2.5. Phenol:chloroform extraction and ethanol precipitation

DNA solutions were extracted by vortexing thoroughly with an equal volume of phenol:chloroform (redistilled phenol (BDH) equilibrated in 50 mM Tris-HCl, pH 8.0, and mixed with chloroform in a 1:1 ratio). After spinning at 14,000 rpm in a bench centrifuge for 10 min at room temperature (RT), the aqueous layer was transferred to a fresh microfuge tube. The extraction was repeated as necessary. DNA was recovered by precipitation with 0.1 volume 3 M sodium acetate, pH 4.6, and 2.5 volumes of ice-cold ethanol, incubated on ice for 15 min, followed by centrifugation at 14,000 rpm for 15 min at RT. The DNA pellet was washed in 70% ethanol, dried and resuspended in the appropriate buffer.

2.2.6. Transfer of nucleic acids from gels to nylon membranes

2.2.6.1. Southern transfer

PCR reaction mixtures (in 4-8 µl) were separated by electrophoresis on a 2% agarose minigel (See 2.2.3). The gel was denatured for 15 min in 100 ml denaturing solution (1.5 M NaCl, 0.5 M NaOH) and rinsed in 10xSSC (20xSSC: 3 M NaCl, 0.3 M trisodium citrate, pH 7.2) for 2 min. DNA was transferred from the gels to Hybond N+ membrane (Amersham) by Southern transfer (Southern, 1975) for 4 h to overnight using 10xSSC as transfer buffer. DNA was fixed to the membranes by contact with a pad of Whatman 3MM paper soaked with 0.4 M NaOH for 20 min and subsequently neutralized by rinsing in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA) for 10 min. Membranes were kept in 2xSSC at 4°C until use.

2.2.6.2. Northern transfer

Nucleic acids, separated by 5% denaturing PAGE (7 M urea) (See 2.2.2.2), were denatured in 0.05 M NaOH for 10 min, rinsed in 0.5XTBE buffer and transferred to positively-charged Hybond N+ membranes (Amersham) in a Mini trans-blot electrophoretic transfer cell (BIO-RAD), with cooling, in 0.5xTBE buffer at 80 V for 1

hour. The RNA was fixed to the membranes by cross linking in the Gene Linker UV chamber (BIO-RAD), using the CL program.

2.2.7. Preparation of plasmid DNA

Small amounts of plasmid DNA were prepared essentially by the following procedure from plasmid-containing bacterial cultures in a stationary phase. An overnight bacterial culture (1.5 ml) grown in 2YT medium and supplemented with the appropriate antibiotics was pelleted by centrifugation at full speed at RT for 1 min in a bench centrifuge. The supernatant was discarded and the cells were resuspended in 90 µl of TES buffer (25 mM Tris HCl, pH 8.0, 10 mM EDTA, 15% sucrose). A solution of freshly prepared 0.2 M NaOH, 1% SDS (180 µl) was added and mixed gently by inversion of the tube. A solution of 3 M NaOH (135 µl), pH 4.6, was added, mixed by inversion and incubated on ice for 3-5 min. The mixture was centrifuged for 20 min at RT and the supernatant transferred to a fresh tube. The DNA solution was treated with 2 µl RNase (10mg/ml) at 37°C for 30 min, followed by extraction with an equal volume of phenol:chloroform (See 2.2.5.). DNA in the aqueous phase was precipitated by the addition of 1 ml ice cold ethanol and pelleted by centrifugation for 20 min as previously. The pellet was washed in 400 µl 70% ethanol, air dried at RT for 15 min and resuspended in 20 µl TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The plasmid DNA was further purified, where necessary, by passage through a Sepharose CL-6B spin column (See 2.2.4).

Larger scale preparations (50 ml) of plasmid DNAs for use as probes were carried out by Ms Halina Bogacz by scaling-up the procedure described above .

2.2.8. Preparation of M13 single-stranded template DNA

A single colony of *E. coli* XL1-B was inoculated into LB-broth (supplemented with the appropriate antibiotic) and incubated overnight at 37°C with continuous shaking. M13 DNA clone in glycerol (10 µl) was inoculated together with 500 µl of this fresh overnight culture of *E. coli* XL1-B into 5 ml of 2YT. The culture was incubated at 37°C

with vigorous shaking for 5 h. Cells were pelleted by centrifugation at 14,000 rpm for 5 min. The supernatant was carefully transferred to a fresh tube and recentrifuged to remove all bacterial cells. To each 1.2 ml aliquot of the supernatant, 200 µl of 2.5 M NaCl, 20% ($^{W}/_{V}$) PEG 6000 (BDH) was added. After incubation for 15 min at RT, the phage pellet was precipitated by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 5 min. All traces of the supernatant were removed with a micropipette, and the pellet was resuspended in 100 µl of TE (10 mM Tris-HCl, pH 8.0, 0.1mM EDTA) buffer and extracted with an equal volume of phenol saturated with TE buffer. The aqueous phase was extracted three times with 500 µl of diethyl ether and ethanol precipitated. The phage DNA was collected by centrifugation, washed in 70% ethanol, air dried, resuspended in 25 µl TE buffer, and stored at -20°C.

2.2.9. Cloning of PCR products

2.2.9.1. Ligation of vector and insert DNAs

Vectors used for cloning were pGEM 5Zf$^+$ or pBluescript SK$^+$ restricted with *Eco* RV (See 2.2.1) or T vectors prepared from *Eco* RV-restricted pBSK$^+$ or pGem 5 Zf$^+$ essentially by the method of Marchuk et al. (1991).

Ligation was carried out in a 10 µl volume with 20-50 ng of vector DNA and the DNA insert fragment in molar ratios of 1:3, 1:2 and 1:1 respectively in a ligation reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 1-2 U T4 DNA ligase (Bresatec, Australia). The reactions were incubated overnight at 4°C. A control ligation with vector only was set up and included in the transformation step to determine background levels of undigested or recircularized vector DNA.

2.2.9.2. Transformation of *E. coli* with plasmids

Freshly prepared competent cells were prepared for each transformation. A 5 ml culture of *E. coli* DH5α was prepared by inoculating a single colony into LB-broth (supplemented with the appropriate antibiotic) and incubating the culture overnight at 37°C with continuous shaking. This culture was diluted 100-fold into 50 ml LB medium

(and antibiotic) and grown at 37°C with constant shaking until the log phase was reached (A_{600}: 0.4-0.6). The cells were centrifuged at 2000 rpm in a Sorvall HB4 rotor at 4°C for 5 min. After draining, the pellets were resuspended in 2.5 ml of ice cold 0.2 M $MgCl_2$ and 0.5 M $CaCl_2$ and incubated on ice for 60 min. 200 µl aliquots of this cell suspension were mixed with 2-5 µl of the DNA ligation reaction mix (See 2.2.9.1) and left on ice for 40 min. The cells were heat shocked at 42°C for 2 min in a water bath. 2YT media (100 µl) was added to the cell suspension which was incubated at 37°C for 30 min to allow the cells to recover. Fifteen µl of 60 mg/ml BCIG (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 20 µl of 20 mg/ml IPTG (isopropylthio-β-D-galactoside) were added to each reaction tube containing the transformed cell suspension which was then plated directly onto LB agar plates supplemented with 100 µg/ml of ampicillin. After overnight incubation at 37°C, white recombinant colonies were selected and used for the preparation of plasmid DNA (See 2.2.7). The orientation of the inserts was determined by restriction digest analysis of the plasmid DNA (See 2.2.1).

2.2.10. DNA sequencing

The dideoxynucleotide chain termination sequencing method (Sänger et al.,1980) was used to determine DNA sequence. DNA sequencing was performed using T7 DNA polymerase on double-stranded plasmid DNAs and sequencing kits supplied by Pharmacia. Plasmid DNAs, purified on a small scale as described in (2.2.7), were denatured by the addition of 4 µl of 2 M NaOH to 16 µl plasmid DNA. Denatured DNAs were purified by passage through Sepharose CL-6B spin columns (See 2.2.4). Ten µl of this purified and denatured DNA was used in each sequencing reaction.

Alternatively, sequencing reactions for automated analysis were carried out by Dr Neil Shirley using the ABI *Taq* DyeDeoxy Terminator Cycle Sequencing kit protocol (Applied Biosystems, USA).

George & Sherrington's book covered many many techniques. Must be more precise.

2.3. General *in vitro* culture techniques

Standard sterile procedures and *in vitro* culture techniques were performed essentially according to procedures recommended by George and Sherrington (1984). Specific modifications of standard techniques are described in the relevant sections.

2.3.1. Standard surface-sterilization procedures

Young shoots, shoot apices and nodal segments were excised from selected varieties of vines grown under greenhouse and field conditions. Explants were carefully cleaned with tap water containing 0.1% ($^{V}/_{V}$) Tween 20 (Sigma) using a small paintbrush to dislodge soil and dust particles. They were then washed in running tap water for 40-60 min. Explants were agitated in a 0.6% ($^{W}/_{V}$) solution of Benlate® DF fungicide (Du Pont de Nemours & Co., Delaware, USA) for 30-60 min before starting sterile procedures.

Surface-sterilization was carried out in 80% ethanol for 30 s followed by 10%-25% ($^{V}/_{V}$) White King bleach (0.4-1.0% available chlorine) containing 0.1% ($^{V}/_{V}$) Tween 20 for 5-15 min depending on the type of explant. Explants were rinsed at least three times in sterile distilled water (SDW) including at least 10 min on a shaker at 90 revolutions per minute (rpm).

2.3.2. Media preparation

Culture media were prepared according to the formulations used. All media were solidified with 6.5-7.2 g/L Bacto agar (Difco) except where specified otherwise, adjusted to pH 5.8 with KOH and autoclaved at 121°C for 20 min. Organic constituents and plant growth regulators (Sigma) were filter-sterilised and added aseptically to the sterile media after the temperature had dropped to 80°C. Media were dispensed into sterile containers, 35 ml polycarbonate culture tubes (Technoplast, Adelaide), 125 ml, 250 ml and 500 ml polycarbonate culture tubs with polypropylene lids (Disposable Products, South Australia).

2.3.3. Incubation conditions

Cultures were routinely incubated in illuminated conditions under cool white fluorescent light (Philips, TLD 36W/33), unless otherwise indicated. The total energy at the explant level was 200-350 μE $m^{-2}s^{-1}$. The photoperiod used was 16h light/8h dark. Temperatures varied from 21°C in the dark to 26±2°C in the light.

2.4. Growth of vines in the greenhouse

2.4.1. Potting mixes and plant growth conditions

Potting mixes were prepared by the Plant Growth Services, University of Adelaide, Waite Campus. Two types of potting mixtures were used (1) recycled soil and (2) UC (University of California) potting mix consisting of four parts washed river sand and three parts (dry volume) moss peat; the latter was used when a consistent potting mix was required. Both types of mixes were steam-sterilised at 100°C for 30 min before use. Plants were grown in 13-25 cm diameter plastic pots under normal glasshouse conditions at 18-25°C, unless otherwise indicated.

2.4.2. Conditioning of *in vitro* plantlets

Rooted shoots were transferred to the greenhouse and conditioned by Ms Carol Smith in the containment glasshouse at the Waite Campus. Vines were planted individually in a mixture of vermiculite, isolite and perlite (1:1:1) in small plastic pots (5x5cm, 6 cm high). The vines were kept under high humidity by placing the pots in a plastic tray filled up to 2 cm deep with water and inverting a standard 500 ml culture tub (Disposable Products, Adelaide) over each pot. After 3 weeks, the vines were transplanted into recycled soil in plastic pots (12.5 cm diameter) with culture tubs inverted over the vines. After 2-3 weeks, the vines were gradually exposed to the atmosphere in normal greenhouse conditions. The vines were fertilised once every 3 weeks with Aquasol as per the manufacturer's recommendations.

Chapter Three

Development of a High Sensitivity RT-PCR Assay for the Diagnosis of Viroids in Grapevines

3.1. Introduction

The detection of viroids in grapevines is difficult compared to other host plants because of the generally symptomless presence of the viroids in grapevines. Of the five viroids known to occur in grapevines, only GYSV1 and GYSV2 have been reported to cause grapevine yellow speckle disease (GYS), independently, in hot weather (Koltunow et al., 1989).

Biological indexing, used until recently (Habili et al., 1992), has been of very limited efficiency because it can only assay for symptoms of yellow speckle (Habili et al., 1992) without distinguishing between the two causal agents. Additionally, the nature of the technique precludes the detection of viroids prior to symptom expression. Serological methods, widely used for the diagnosis of viruses, are not applicable to viroids because of the absence of a protein coat.

Hybridization analysis using labelled probes has been used extensively for the detection of viroids because of the high specificity and sensitivity achievable. The five grapevine viroids have been detected by this technique, GYSV1, GYSV2 and HSV directly in grapevine tissue and AGV and CEV, the two viroids in lower titre, after multiplication in an alternative host. Differentiation between GYSV1 and GYSV2 was not always possible because of the high sequence homology between these two related viroids, which resulted in cross-hybridization. Also, more sensitive viroid detection techniques are required because of fluctuations in viroid load which are not always detectable by methods currently in use (Morton et al., 1993; Gafny et al., 1995).

Reverse transcription and polymerase chain reaction (RT-PCR) techniques have been shown to improve dramatically the sensitivity of detection of other pathogens (See 1.8.5). However, RT-PCR has not been much used for viroid indexing. A few applications of RT-PCR to viroids in grapevines have been reported (Rezaian et al., 1992; Staub et al., 1995a). The first successful application of RT-PCR to grapevine viroids was reported by Rezaian et al. (1992) and it enabled the differentiation between the closely related GYSV1 and GYSV2 viroids. However, a low efficiency of amplification was obtained, requiring the additional procedure of radioactive detection of the PCR

products. Subsequently, Staub et al. (1995a) reported a more efficient RT-PCR assay that allowed the simple detection of the PCR products on an agarose gel after ethidium bromide staining.

A prerequisite for RT-PCR analysis in grapevines is an efficient viroid extraction procedure. Grapevines are notorious for their high levels of phenolic compounds, polysaccharides and other complex substances that have inhibitory effects on RT-PCR enzymes (Rezaian et al., 1992; Rowhani et al., 1993; Levy et al., 1994; Minafra and Hadidi, 1994; Staub et al., 1995a).

The development of RT-PCR assays is frequently carried out using samples known to be positive by other diagnostic methods. Samples assaying negatively are often "spiked" with viroid-positive material and the resulting positive reaction is commonly interpreted as evidence of their negative viroid status. However, this may instead only demonstrate the absence of inhibitors of the RT-PCR reaction in the RNA extract. In addition, it may also reflect the limit of sensitivity of the amplification conditions. Hence, the use of a protocol developed on a highly infected sample may not be directly applicable to samples at a lower level of infection. Low viroid load samples require the optimisation of PCR reaction conditions for the highly specific amplification of the low copy number templates to a level detectable over the vast background of host nucleic acids.

The objective of the work described in this chapter was to develop a simple and efficient protocol for the extraction of RNA from grapevine leaves and a high sensitivity RT-PCR assay that is reliable for the routine diagnosis of the five grapevine viroids.

3.2. Materials and Methods

3.2.1. Sources of plant material

V. vinifera varieties, Riesling, Sauvignon Blanc, Chardonnay, Grenache, Colombard, Pinot Noir, Mataro, Pedro Ximenes and Shiraz 12 were obtained from the field collection of the Department of Horticulture, Viticulture and Oenology, Waite Campus, University of Adelaide, South Australia. Shiraz BVRC 30 was obtained from Orlando Wyndham, Barossa Valley. The Sultana H5 and the Emperor seedling Vfr were

kindly supplied by Mr. L. Krake, CSIRO Division of Horticulture, Adelaide. The Vfr seedling (Koltunow and Rezaian, 1988) was maintained in an isolated area under normal glasshouse conditions. Vfr samples used in this thesis were obtained from vines regenerated from cuttings of the original Vfr vine and maintained in the containment glasshouse, Waite Campus, since 1993. The Emperor seedling RTG was germinated aseptically on culture medium and maintained *in vitro* to avoid contamination by external sources.

The four low viroid load vines were Cabernet Sauvignon (CS) vines, CS R6V4/9, CS R6V4/10, CS R15V1/5 and CS R15V1/8, regenerated by shoot apical meristem culture (SAMC). They were produced Dr. G. Burrows and Mr. K. Ashton, Charles Sturt University, WaggaWagga, New South Wales, by a method (Burrows and Ashton, 1993) modified from Barlass and Skene (1978), as part of the Cooperative Research Centre for Viticulture Subprogram 1.4: Tissue Culture and Molecular Genetic Manipulation of Grapevine. Leaf samples were obtained from the SAMC-derived vines in the shadehouse and from their stock vines in the fields and kept at -80°C.

The negative control vines used in dot blot analysis were: for GYSV1, two CS vines regenerated by SAMC, CS 125/R2A2S13 and CS 125/R2A8S2, and for AGV, two rootstocks, Dog Ridge A6V8 and Paulsen 1103. All four vines were obtained through the kind assistance of Mr. G. Fletcher, Victoria and Murray Valley Vine Improvement Association (VAMVIA), Irymple, Victoria.

3.2.2. RNA extraction for viroids

RNA extraction was carried out using a modification of Method 4 from Rowhani et al. (1993). Young, newly-expanded grapevine leaf tissue (1.0 g) was ground in a cold mortar in 10 ml of extraction buffer (95 mM K_2HPO_4, 95 mM KH_2PO_4, 10% ($^{W}/_{V}$) sucrose, 0.15% ($^{W}/_{V}$) bovine serum albumin (BSA fraction V, Sigma), 2% ($^{W}/_{V}$) polyvinylpyrrolidone (PVP-10, Sigma), 0.53% ($^{W}/_{V}$) ascorbic acid), adjusted to pH 7.6 before extraction. The leaf suspension, in a 10 ml tube, was centrifuged at low speed (75-100 g) for 2 min in a bench centrifuge. The supernatant was transferred to a 15 ml

Corex tube and spun at 11,000 rpm for 10 min. The pellet obtained was resuspended in 2ml TE_1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) to which 0.1% (v/v) 2-mercaptoethanol and 0.65% ($^{W}/_{V}$) sodium sulphite were added to prevent browning. SDS (250 µl of a 10% solution) was added to the suspension which was dispensed into two microfuge tubes and incubated for 10 min at 60°C. The tubes were cooled on ice for 1 min and spun at 14,000 rpm on a bench centrifuge at 4°C for 5 min. The supernatants were transferred to two fresh microfuge tubes and incubated with 400 µl of 5 M potassium acetate at -20°C for 10 min. The tubes were centrifuged again at 14,000 rpm for 10 min and the nucleic acids in the supernatant precipitated by incubation with a 0.1 volume of 3 M sodium acetate (pH 5.2) and a 0.8 volume of ice cold isopropanol for 30 min at -80°C. The nucleic acids, recovered by centrifugation at 14,000 rpm for 20 min at 4°C, were resuspended in 500 µl TE_2 buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) before precipitation with 2M NaCl and one volume isopropanol. The pellet was washed in 70% ($^{V}/_{V}$) ethanol, resuspended in TE_2 buffer and stored at -80°C. The absorbance spectrum of the the total nucleic acid extract (A_{210}-A_{320nm}) was read at this stage and the concentration of nucleic acids calculated from the optical density at 260 nm. All quantitative estimations used in subsequent work were based on these data.

3.2.3. DNase treatment

The total nucleic acid extract was digested with RNase-free DNase (RQ-1 grade, Promega) before RT-PCR studies using the Promega protocol. Total nucleic acids (50 µg) were treated with DNase (1U/ 3 µg nucleic acid) in a 100 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 200 U rRNasin (Promega) at 37°C for 30 min followed by incubation at 95°C for 4 min to inactivate the DNase. RNA was recovered by precipitation with a 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol and resuspended in TE_3 buffer (10 mM Tris-HC1, pH 7.5, 0.1 mM EDTA) to give a concentration of 1 µg/µl nucleic acid.

3.2.4. Oligonucleotide primers

Primers designed to the sequences of all five viroids (Rezaian et al., 1992; Visvader and Symons, 1985) were used initially to give mostly full-length viroid PCR products. New primers were subsequently designed with the help of Oligo 4 primer analysis software (National Biosciences, USA) to give partial-length PCR products less than 140 bp and were synthesised by Dr Neil Shirley (See 2.1.1). The primer pairs used for each viroid, their parameters of amplification and the sizes of the expected products are given in Table 3.1. To differentiate between the two sets of primers, they are referred to in the text as full-length and partial-length primers. Two additional GYSV2 primer pairs, c2h1 and c1h2, giving PCR products of 194 and 231 bp respectively were also used. The positions of all the primers used in this study in relation to the five viroid domains are shown in Fig. 3.1.

3.2.5. Reverse transcription of viroid RNA

Viroid RNA (in 4 µg extract, DNase treated) was annealed to 6 pmoles of the reverse primers by heating at 95°C for 3 min and snap-cooling on ice for 1 min. Reverse transcription was carried out in a 25 µl reaction volume containing 1xbuffer supplied with the enzyme (50 mM KCl, 10 mM Tris-HCl, pH 8.8, 0.1% ($^{v}/_{v}$)Triton X-100), 4 mM dithiothreitol (DTT), 1 mM of each of the four dNTPs (Pharmacia) and 2 U Avian Myeloblastosis Virus reverse transcriptase (AMVRT, Promega) and incubated at 52°C for 30 min. The reaction was stopped by heating to 95°C for 4 min to denature the reverse transcriptase.

3.2.6. PCR amplification of cDNA

3.2.6.1. Standard PCR protocol

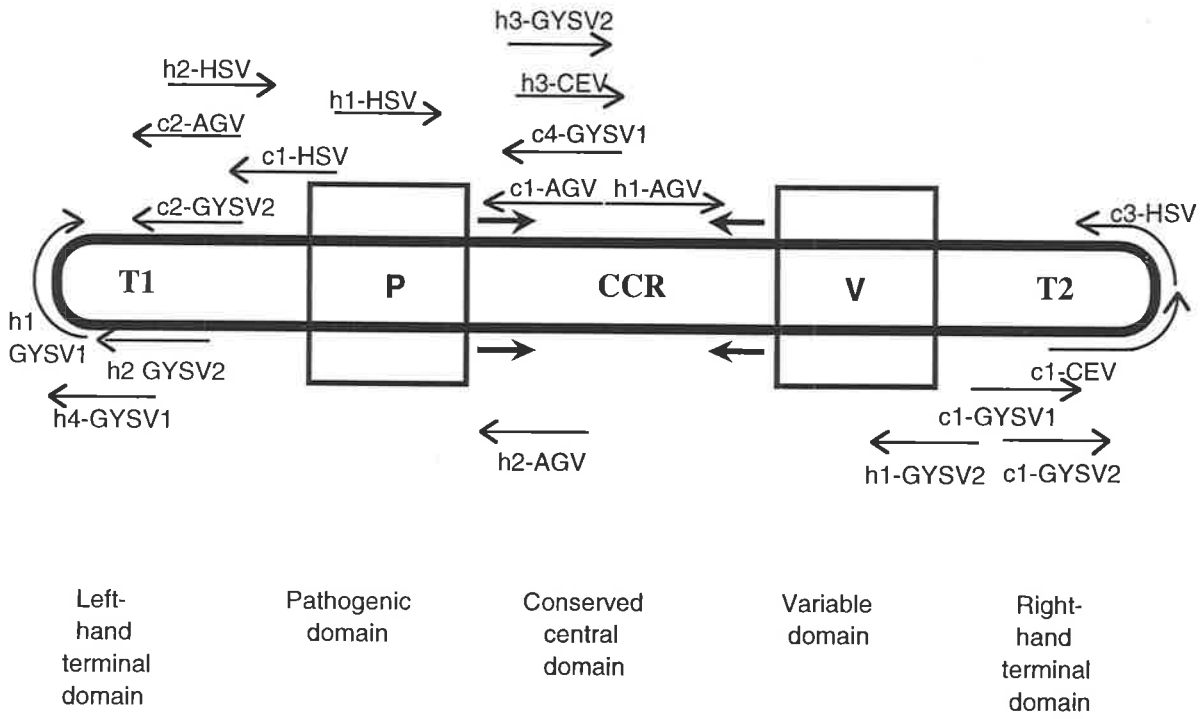
The amplification reactions were set up in 0.5 ml microfuge tubes as follows: 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100) supplied with the Promega enzyme, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1 µl of reverse transcription mixture and 1U of *Taq* DNA polymerase (Promega). A layer

Table 3.1: Primers used for RT-PCR studies on viroids and their parameters for optimal amplification

Viroid	Primer	Sequence	Position	Product size bp	No. cycles	Annealing Temp.(T_a)^oC
GYSV1	c1 -	5'GCG AAC GTG AAT AGG CTA GAA 3'[a]	214-194	223	30	56
	h1 +	5'GCT AAG AGG TCT CCG GA 3'[a]	359-8			
	c4 -	5'CGA CGA CGA GGC TCA CT 3'	105-85	120	30	58
	h4 +	5'ACC CCC GCT AAG AGG TC 3'	353-2			
GYSV2	c1 -	5'ACC GGC TTC GGA GAT AGA AG 3'[a]	208-189	123	35	56
	h3 +	5'AAT GAG CCT CGT CGT CGA 3'	86-104			
	c2 -	5' CCG AGG TGT AAC CAC AGG AAC A 3'	39-18	194	25	55
	h1 +	5' TTG AGG CCC GGC GAA ACG C 3'[a]	209-227			
	h2 +	5'GAA CCC CTG CGA AAA AGG 3'	341-358	231*	35*	55*
HSV	c1 -	5'TTG CTT GCC TGA TGC GGC A 3'[a]	36-18	297	30	56
	h1 +	5'AGA AAA AAC AAG GCA GG 3'[a]	37-53			
	c3 -	5'AGC CTC TAC TCC AGA GCA 3'	148-131	138	25	56
	h2 +	5'CTC GAG TTG CCG CAT CAG G 3'	11-29			
AGV	c2 -	5'TTC GGT GAG TAC CAC AGG AAC 3'	37-17	130	35	55
	h2 +	5'ACT CGT CCC AGC GGT CCC AAC 3'	277-297			
	c1 -	5'GTC GAC GAC GAG TCG CCA GGT GAG TCT T 3'[a]	78-105	375	35	59
	h1 +	5'GTC GAC GAA GGG TCC TCA GCA GAG CAC C 3'[a]	100-127			
CEV	c1 -	5'CGA AAG GAA GGA GAC GAG CTC CTG 3'[b]	198-175	115	35	59
	h3 +	5'TTC AGG GAT CCC CGG GGA A 3'	84-102			

[a] Rezaian et al., 1992; [b] Visvader and Symons, 1985; * for c1h2 GYSV2 primer pair.

Fig. 3.1: The position of the primers used for RT-PCR studies in relation to viroid structure



The arrows in bold indicate the position of the inverted repeats in the conserved domain

The other arrows indicate the position and orientation of the primers listed in Table 3.1

of sterile paraffin oil (20 µl, Sigma) was added on top of the reaction mixtures. Amplification was carried out in an MJ minicycler (MJ Research Inc., USA, model PTC 150) using the following protocol: 94°C for 1 min, annealing temperature (T_a) °C for 1 min, 74°C for 1 min, for 20-30 cycles.

3.2.6.2. Optimised PCR protocol for fast cycle times

The buffer composition and the reaction components used for amplification by the optimised protocol for fast cycle times were based on that developed by Wittwer and Garling (1991). Viroid cDNA amplification was performed in a 20 µl reaction mixture containing 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM $MgCl_2$, 250 µg/ml BSA, 0.3 mM each dNTPs, and one unit of *Taq* DNA polymerase (Promega). A bulk amplification mixture of the buffer, $MgCl_2$, dNTPs and BSA (Bresatec, Australia) was prepared and kept frozen in aliquots to minimise variation between assays and to reduce the chances of contamination. The cDNA, in 1 µl of the reverse transcription mixture, was annealed to 6 pmoles of each of the two primers (Table 3.1) by incubating at 94°C for 2-3 min in a heating block and snap-cooling on ice before adding the other reaction components. The reaction mixture was then loaded into positive displacement plastic capillary tips (Tri-Continent, USA) and sealed individually in a Bunsen flame.

PCR was carried out either in an MJ minicycler (MJ Research Inc., USA, model PTC150 adapted for 36 capillary tubes) or in a Corbett Research FTS-1 thermal cycler, using the following standard protocol: 74°C for 40 s, followed by 94°C for 15 s, T_a°C for 15 s and 74°C for 40 s for the first five cycles, followed by 94°C for 5 s, T_a°C for 5 s and 74°C for 20 s for the remainder of the cycles and a final elongation of 5 min at 74°C. For the first five cycles, a T_a 5°C lower than that specified in Table 3.1 was used.

PCR reaction products were separated by electrophoresis on 2% agarose gels or 4% agarose gels for PCR products smaller than 120 bp (See 2.2.2.1).

3.2.6.3. Protocol for combined RT-PCR (cRT-PCR)

Combined RT-PCR in a one tube system was carried out essentially according to the method described by Sellner et al. (1992). The reaction was performed in a microfuge tube (0.5 ml) in a 25 µl reaction volume containing 1x*Taq* polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100) supplied with the Promega enzyme, 2 mM $MgCl_2$, 1 mM β–mercaptoethanol, 6 µM EDTA, 0.2 mM each dNTPs, 0.2 µM of each primer, 0.5 U of AMVRT (Promega) and two units of *Taq* DNA polymerase (Promega), under a layer of sterile paraffin oil (Sigma). cRT-PCR was carried out using the following protocol: 52°C for 36 min for reverse transcription, 94°C for 4 min for the denaturation of AMVRT, followed immediately by the amplification steps of 94°C for 1 min, T_a°C for 1 min, 74°C for 1 min for the specified number of cycles, followed by a final elongation of 5 min at 74°C. For the first five cycles, a T_a 5°C lower than that specified in Table 3.1 was used.

3.2.6.4. Protocol for cRT-PCR for fast cycle times

The combined RT-PCR protocol in a one tube system was adapted for amplification by fast cycle times in a capillary tip. Viroid RNA (in 160 ng non-DNAse-treated nucleic acid) was annealed to 6 pmoles of each of the two primers as described in Section 3.2.4.3. The reaction was performed in a 20 µl reaction buffer containing 50 mM Tris-HCl, pH 8.5, 20 mM KCl, 3 mM $MgCl_2$, 1 mM β–mercaptoethanol, 6 µM EDTA, 250 µg/ml BSA, 0.5 mM each dNTPs, 0.5 U of AMVRT (Promega) and two units of *Taq* DNA polymerase (Promega). cRT-PCR was carried out in the MJ minicycler for capillary tips using the following protocol: 52°C for 36 min, 94°C for 4 min for reverse transcription and denaturation of the AMVRT respectively, followed immediately by the amplification steps 94°C for 15 s, (T_a) for 15 s and 74°C for 40 s for the first five cycles, followed by 94°C for 5 s, T_a°C for 5 s and 74°C for 20 s for the remainder of the cycles and a final elongation of 5 min at 74°C. For the first five cycles, a T_a 5°C lower than that specified in Table 3.1 was used.

3.2.6.5. Precautions against contamination by exogenous templates

Mortars and pestles were treated with 10% ($^{W}/_{V}$) potassium hydroxide for 10 min and rinsed copiously with autoclaved water before extractions were performed. Contamination at the PCR stage was eliminated by a number of precautions as reviewed by Yap et al. (1994). Reagents were dispensed in as small aliquots as practical, for a single use wherever possible. Reaction mixtures were set up in a separate laboratory in a UV-sterilised laminar flow hood, without the air flow on, and using a set of micropipettes kept exclusively for this purpose. Contamination by aerosols containing sample DNA was prevented by the use of filter tips (Robbins Scientific, USA). Capillary tubes were individually sealed in a Bunsen flame since a capillary tube sealing apparatus was found to be a major source of contamination. The contents of the positive displacement capillary tubes were transferred into microfuge tubes by centrifugation, after removal of the plunger.

3.2.7. Viroid cDNA clones

All five viroid cDNA clones contained full-length monomeric inserts. The two yellow speckle clones were obtained from Dr. A. Rezaian, CSIRO Division of Horticulture, Adelaide. The GYSV1 clone 373A contained a GYSV1 insert in the *Eco* RI site of pBluescript SK+. The GYSV2 clone contained a plus sense sequence in the *Bam*H1/*Kpn*1 site of the bacteriophage M13 mp19. The CEV clone P12, constructed by Dr. A. Rakowski in our laboratory, contained a CEV A insert in the *Pst* I site of pGEM2. The HSV cDNA clone was constructed by cloning PCR products, amplified from c1h1 HSV primers (Table 3.1), into *Eco* RV site of pBluescript SK+ by Mr. D. Webb, also in our laboratory.

The AGV cDNA clone was obtained as follows: full-length AGV sequences were obtained by amplification from Sultana H5 RNA extracts by the standard protocol for fast cycle times using the high-fidelity Vent® DNA Polymerase (New England Biolabs, USA), c1h1 AGV primers and the parameters defined in Table 3.1. The required fragment, after separation of the PCR products on a 2% agarose gel (See 2.2.2) and

purification (See 2.2.3), was cloned into pBluescript SK+ vector cut with *Eco* RV (See 2.2.9) The orientation of the clone was confirmed by restriction and sequence analysis (See 2.2.10).

3.2.8. Preparation of ^{32}P-labelled cDNA probes

Viroid cDNA clones were prepared as described in sections 2.2.7 and 2.2.8. Viroid cDNA inserts were amplified by PCR from viroid cDNA clones using M13 forward and reverse sequencing primers, or primers complementary to the SP6 and T7 RNA polymerase promoters. The oligonucleotide primers, which were synthesised by Dr Neil Shirley (See 2.1.1), were M13-40P (5' CAG GGT TTT CCC AGT CAC GAC 3') and M13-RSP (5' ACA GGA AAC AGC TAT GAC CAT G 3') for clones in pBluescript SK+ plasmid vector and M13 bacteriophage vector or the primers SP6 (5' GAT TTA GGT GAC ACT ATA G 3') and T7 (5' TAA TAC GAC TCA CTA TAG GG 3') for clones in pGEM-2. DNA fragments of interest were excised from the plasmids by digestion with the appropriate restriction enzyme (See 2.2.1) and purified (See 2.2.3) after separation of the fragments on agarose gel (See 2.2.2).

^{32}P-labelled cDNA probes were prepared from these purified viroid cDNA inserts essentially by the random priming method of Feinberg and Vogelstein, (1983) and purified by the method of Collins et al. (1996). The DNA templates (20 ng) were denatured in the presence of 200 pmoles of random-sequence 9-mer oligonucleotide primers in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by heating to 95°C for 2 min, followed by cooling on ice for 5 min. The labelling reaction was carried out overnight at RT in a 25 µl reaction volume in 1x labelling buffer (2.5xlabelling buffer was 0.5 M HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid), pH 6.6, 0.125 M Tris-HCl, pH 8.0, 12.5 mM $MgCl_2$, 12.5 mM DTT, 1.0 mg/ml BSA), 30 µCi α-^{32}P-dCTP, 20 µM each dATP, dGTP and dTTP and 1 U *E. coli* DNA polymerase I (Klenow fragment).

Unincorporated dNTPs were removed by passage through Biogel P-10 (BIO-RAD) spin columns prepared as follows: 1.0 ml syringes, with plungers removed, were

plugged at the bottom with sterile glass wool and filled to the top with Biogel P-10 equilibrated in TEN buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.0 mM EDTA) and centrifuged at 3000 g for 4 min to remove excess TEN buffer. Probe reaction mixtures were diluted to 200 µl with TEN buffer and passed through the column by centrifugation at 3000 g for 4 min.

3.2.9. Southern hybridization analysis with cDNA probes

PCR products, separated on a 2% agarose gel, were transferred to Hybond N+ membranes (Amersham) by Southern transfer (Southern, 1975) (See 2.2.6.1). Hybridization analysis was carried out using ^{32}P-dCTP-labelled cDNA probes prepared from viroid cDNA clones not containing primer sequences.

The prehybridization and hybridization procedures used were based on the protocols used by Collins et al. (1996). The prehybridization solution was made up as follows: 3 ml 5xHSB solution (3 M NaCl, 100 mM PIPES (1, 4-Piperazine diethanesulfonic acid), 25 mM Na_2 EDTA, pH 6.8), 2 ml 50xDenhardt's solution, (2% ($^{W}/_{V}$) Ficoll 400 (Pharmacia), 2% ($^{W}/_{V}$) PVP (Sigma), 2% ($^{W}/_{V}$) BSA fraction V (Sigma), 3 ml 25% ($^{W}/_{V}$) dextran sulphate, 2 ml MilliQ H_2O and 200 µl salmon sperm DNA (10 mg/ml) sheared and denatured by heating at 95°C for 3 min prior to addition. Membranes were prehybridised in 5-20 ml of the prehybridization solution in a hybridization bottle at 60°C for 2-3 h. Labelled probes, denatured by heating at 95°C for 3 min, were added to the prehybridization solution and hybridization carried out overnight at 65°C. The hybridization solution was then discarded and the filter washed as follows: twice in 2xSSC (1xSSC is 0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.2) , 0.1% SDS at RT for 5 min; twice in 0.2xSSC, 0.1% ($^{W}/_{V}$) SDS at 65°C for 10 min and a final wash 0.2xSSC, 0.1% SDS preheated to 65°C; this wash was continued until the temperature reached RT. Membranes were blotted dry and exposed for 1-3 days to X-ray film (Fuji RX) at -80°C using an intensifying screen.

3.2.10. Preparation of dot blots

Samples (4 µg nucleic acid, non DNase-treated) were resuspended in 4 µl of a denaturing buffer solution (50% deionized formamide, 1xMOPS buffer (50 mM 3-(N-morpholino) propanesulfonic acid, 1mM EDTA, pH 7.0) and 1xSSC) and heated at 68°C for 15 min before applying to a nylon membrane (Hybond N^+, Amersham). The RNA was then fixed to the membrane by cross linking in the BIO-RAD Gene Linker UV chamber using the CL program.

Samples were prepared for dot blot hybridization analysis on replicate membranes; a separate membrane was used for each of the four viroids assayed. Dot blot samples for each vine were prepared in a single bulk mixture, denatured and applied to the four membranes at the same time. Re-probing of membranes after stripping was not carried out to avoid the loss of signals from low titre samples.

Negative controls for each viroid included nucleic acid extracts from the control vine samples (See 3.2.1), previously found negative for specific viroids by hybridization analysis using ^{32}P-labelled riboprobes, and also sequences of the other grapevine viroids assayed. Sequences of each viroid assayed, as near full-length as possible and derived from amplification by PCR from viroid cDNA clones, were serially-diluted 10-fold starting from 1 ng and used to verify the sensitivity of the probes for each specific viroid.

3.2.11. Preparation of ^{32}P-labelled riboprobes

^{32}P-labelled riboprobes were prepared from the full-length viroid cDNA clones (See 3.2.4) by *in vitro* transcription. Plasmids were linearised by digestion with the appropriate restriction enzymes (See 2.2.1), separated from undigested DNA on agarose gel and purified (See 2.2.3.) before being used as templates. The enzymes used to linearize the cDNA clones were: for GYSV1, *Bam* H1; HSV and CEV, *Xba*1; and AGV, *Eco* R1. Transcription was carried out according to Melton et al. (1984) in a reaction mixture (20 µl) containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each ATP, CTP and GTP, 12 µM UTP, 1 U/µl rRNasin (Promega), 1 µg linearised template, 60 µCi ^{32}P-labelled UTP and 40 U of T7

RNA polymerase (Promega). Transcription reactions were incubated at 37°C for 1 h. The DNA template was destroyed by incubating with 5 U of RNase-free DNase (RQ-1 grade, Promega) for 15 min at 37°C. Transcripts were precipitated by the addition of ammonium acetate to 2.5 M and one volume of isopropanol. The RNA pellet recovered after centrifugation was washed in 70% ethanol, air dried and resuspended in 200 µl TEN buffer. Unincorporated radioactive label remaining after precipitation was removed by passage through a Biogel P-10 spin column (See 3.2.6) before use.

3.2.12. Hybridization with ^{32}P-labelled cRNA probes

Membranes were pre-washed with 0.1xSSC and 0.1% ($^{W}/_{V}$) SDS for 1 h at 67°C and prehybridized for at least 4 h at 65°C in 10 ml hybridization mixture made up with 50% ($^{V}/_{V}$) deionized formamide, 5xSSC, 5xDenhardt's solution, 0.02 M sodium phosphate, pH 6.8, 1% ($^{W}/_{V}$) SDS. Salmon sperm DNA and *E. coli* tRNA, each at a concentration of 100 µg/ml, were denatured by heating at 95°C for 3 min before adding to the mixture. The membranes were hybridised in fresh prehybridization buffer to which ^{32}P-labelled riboprobes (10^6 cpm/ml final concentration) were added after denaturation at 80°C for 1 min in an equal volume of deionized formamide. After overnight hybridization at 68°C, the membranes were rinsed in 0.2xSSC, 0.1% SDS at RT, washed twice in 50 ml 0.2xSSC, 0.1% SDS at 68°C for 20 min and finally in 1.5 L of 0.1xSSC, 0.1% SDS with agitation at 75°C for 60 min. Autoradiography was carried out by exposure to Fuji RX film with an intensifying screen at -80°C for 120 h.

3.3. Results

3.3.1. Viroid RNA extraction

The protocol developed for the extraction of viroids was adapted from the method used by Rowhani et al. (1993) to detect fanleaf virus by RT-PCR in grapevines. Two major modifications were made:

(1) An additional reprecipitation step with NaCl to remove polysaccharides (Fang et al., 1992) known to inhibit DNA polymerases (Shioda and Marakami-Muofushi, 1987).

(2) A DNase treatment step which was found to have a positive effect on the specificity of the expected PCR product.

This extraction protocol, suitable for fresh and frozen tissue, gave an average yield of 250-350 µg/g fresh weight tissue of high quality total nucleic acid (A_{260}/A_{230} ratio of 1.9) (Fig. 3.2) which remained in good condition after storage for more than 2 years at -80°C. DNase treatment (Fig. 3.3) reduced the number of non-specific high molecular weight bands although a reduction of yield of the expected PCR product was observed at the same time, possibly as a result of low level contamination of DNase with some RNase. RT-PCR analysis was carried out successfully on extracts from 19 different grapevine varieties.

3.3.2. Optimisation of RT-PCR by fast cycle times

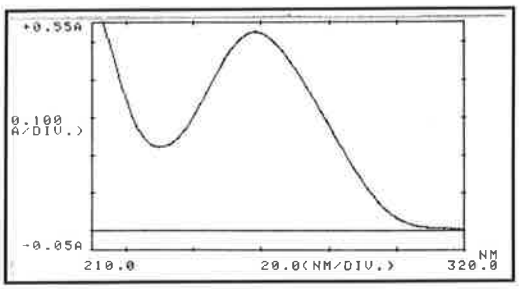
Low viroid load vine samples were obtained from four SAMC-derived Cabernet Sauvignon vines from WaggaWagga for the development of this sensitive RT-PCR assay. The Sultana H5 vine was used as a high viroid load control sample. The viroid loads in these vines were known from the intensity of the hybridization dots obtained by dot blot hybridization analysis (data not shown). HSV and GYSV1 could be detected in the Sultana H5 vine by a standard PCR amplification protocol (See 3.2.4.4.) using primer pairs c1h1 HSV and c1h1 GYSV1 (Table 3.1), but not in the SAMC-derived vines.

3.3.2.1. Selection of primer pairs

Preliminary work using primers giving full-length viroid PCR products had indicated a poor correlation between RT-PCR and dot blot hybridization results, with samples positive by the latter method assaying negative by RT-PCR. New primers were designed in the conserved regions of viroid structure to pick up the targeted viroid population including sequence variants (Keese et al., 1988) and to give a product length of less than 140 bp (Table 3.1). Because the sensitivity of PCR had been reported to vary up to 1000-fold with different primers (He et al., 1994), at least two pairs of primers

Fig. 3.2: Absorbance spectrum (A_{210}-A_{320nm}) of total nucleic acid extracts from a vineyard-grown Chardonnay.

Fig. 3.3: Effect of DNase treatment of nucleic acid extracts on the amplification of HSV. HSV was amplified using c_3h_2 HSV primers and the parameters given in Table 3.1. Samples were, in **lanes 1** and **4** Shiraz BVRC 30/7/15; in **lanes 2** and **5**, Shiraz 12/29; and in **lanes 3** and **6**, Sultana H5. Nucleic acid extracts were in **lanes 1-3**, DNase treated and in **lanes 4-6,** non-DNase-treated. The PCR products (in 5 µl) were separated on a 2% agarose gel. The expected size of the PCR product was 138 bp. The DNA size markers were pUC 19/*Hpa*II fragments.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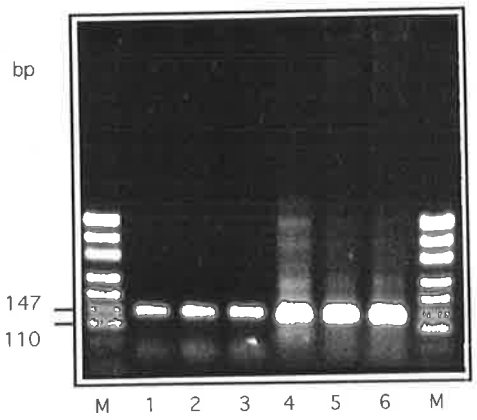
0.100
A/DIV.)
-0.05A
210.0
20.0(NM/DIV.)
320.0
NM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were designed for each viroid. These were tested in all possible combinations to determine the best primer pair in terms of sensitivity as well as specificity. For example, out of four primer pairs used for AGV, the c2h2 AGV primer pair (Fig. 3.4a, lane 4) gave the best amplification compared to the three others (Fig. 3.4a, lanes 1, 2 and 3) and was therefore selected for further optimization. The optimal number of PCR cycles required for the detection of AGV using the c2h2 AGV primer pair was found to be 35 (Fig. 3.4b, lane 4). In general, viroid amplification was found to be highly sensitive to primer position in relation to viroid structure, with a minor shift in the primer position affecting the yield dramatically. Primers giving short PCR product length were found to amplify more efficiently from different vine varieties and results obtained with these primers gave a better correlation with results from dot blot analysis. The most effective primer pairs could be selected only through trial and error.

3.3.2.2. Optimization of the cycle parameters for low copy number templates

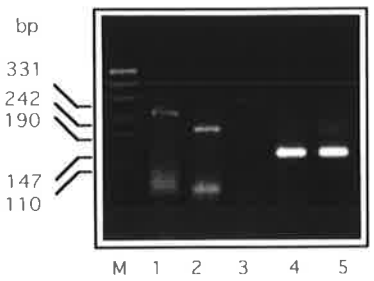
Earlier work by Wittwer and Garling (1991), studying the effects of varying temperatures and cycle times for the amplification of β-globin from human DNA, showed that optimal product specificity and yield were obtained using the extremely short denaturation and annealing times of 1 second or less for each of these two parameters. The rapid temperature control was achieved by the use of 10 µl thin-glass capillary tubes in a specially modified thermal cycler based on recirculating hot air. Work by Douglas and Aitchison (1993) had also indicated that prolonged exposure to 94°C caused a reduction in yield as a result of the degradation of the PCR product. Hence, amplification of viroid cDNA by fast cycle times was chosen in preference to amplification by standard protocols (Sambrook et al., 1989). The cycle times were adapted for use with the more commonly-used plastic positive displacement capillary tips and the thermal cyclers available in the laboratory.

A higher concentration of the following reaction components was required for amplification by fast cycle times as compared to the standard protocol: $MgCl_2$ was

Fig. 3.4: Optimisation of PCR protocol for the detection of AGV. (a) RT-PCR was carried out using four different AGV primer pairs. Vine samples were in **lanes 1-4,** Sultana H5; in **lane 5**, was the AGV cDNA clone as positive control template. The AGV primer pairs were: in **lane 1**, c1h1; **lane 2**, c1h2; **lane 3**, c2h1; **lanes 4** and **5**, c2h2 (Table 3.1). PCR was carried out for 40 cycles using a T_a of 56°C. The expected product sizes in **lanes 1-5** were 375, 198, 307, 130 and 130 bp respectively. PCR products (in 4 µl) were separated on a 2% agarose gel. **(b)** Optimisation of the number of amplification cycles using c2h2 AGV primers and a T_a 55° C. The number of cycles was in **lane 1**, 20; **lane 2,** 25; **lane 3**, 30 and in **lane 4**, 35. The four reaction mixtures were set up at the same time and at the specified number of cycles, one tube was withdrawn from the thermal cycler. PCR products (in 8 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments.

Fig. 3.5: Effect of the initial steps of the PCR protocol on the amplification of GYSV1. The low viroid load vine samples were: in **lanes 1, 4** and **7**, CS R15V1/5; in **lanes 2, 5** and **8**, CS R15V1/8; and in **lanes 3, 6** and **9,** the high viroid load control vine sample, Sultana H5. Assay was carried out using c4h4 GYSV1 primer pair (Table 3.1). Primers were annealed to the templates by heating at 95°C for 2 min and snap-cooling on ice before adding the rest of the reaction mixture and loading into capillary tubes. The first step in the thermocycler was: in **lanes 1-3**, 2 min at 94°C; in **lanes 4-6,** no treatment; in **lanes 7-9**, 40 s at 74°C. The amplification protocol used thereafter was 94°C/15s, 53°C/15 s, 74°C/40 s for 5 cycles, 94°C/5s, 58°C/5 s, 74°C/20 s for 25 cycles and 74°C for 5 min. PCR products (in 4 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments.

(a)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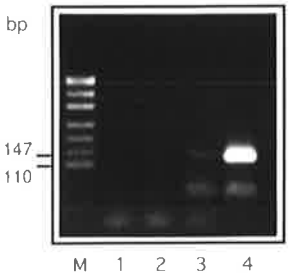
(b)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bp
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M 1 2 3 4 5 6 7 8 9 M

increased from 2 mM to 3 mM, dNTPs from 0.2 mM to 0.3 mM and primers from 0.2 μM to 0.3 μM based on the optimization for each of these reagents. BSA was found essential, presumably because it reduced the surface denaturation of the polymerase over the large surface area of capillary tubes.

In the optimised PCR protocol adapted for plastic capillary tips, two primers were annealed to the viroid cDNA templates by heating to 94°C and snap-cooling on ice before the addition of *Taq* DNA polymerase and other reagents. This first annealing step was carried out manually to obtain a minimal annealing time not obtainable in the thermal cycler because of the long ramping down time (15-20 s). The next step was found to have a marked influence on the ultimate yield and specificity of amplification of the low viroid load templates (Fig. 3.5). A comparison of three conditions showed that the amplification of the cDNA template was more efficient when the primer-annealed templates were taken directly to an extension step of 74°C (Fig. 3.5, lanes 7 and 8) rather than a denaturation step of 2 min (lanes 1 and 2) or when there was no preliminary first step (lanes 4 and 5). These variations in conditions did not affect the product yield and specificity of the high viroid load samples (lanes 3, 6 and 9).

All five viroids were detected by this RT-PCR protocol after the optimization of the annealing temperature and the number of cycles for each selected primer pair. A good correlation with dot blot hybridization results was observed for the higher viroid load samples (Table 3.2). In earlier stages, the PCR products were confirmed by Southern analysis using ^{32}P dCTP-labelled probes prepared from cDNA clones with PCR primer regions excised. In later stages, they were confirmed by restriction enzyme digestion with at least two enzymes (data not shown).

3.3.2.3 Sensitivity of the RT-PCR assay for viroid detection compared to the dot blot hybridization assay

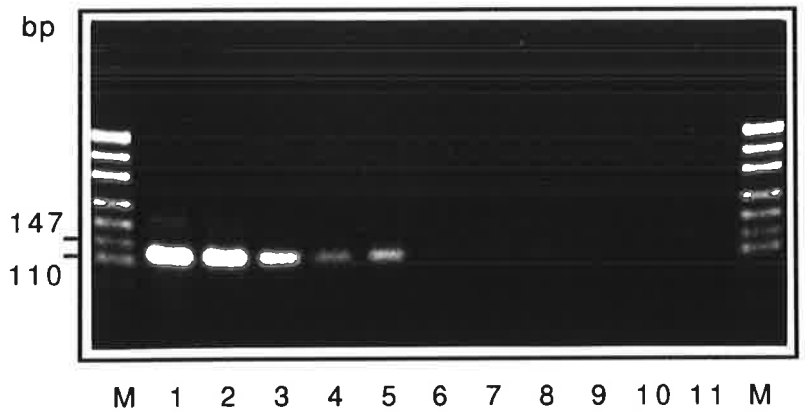
RT-PCR and dot blot hybridization assays were carried out for GYSV1 on serial 10-fold dilutions of total RNA extracts from the high viroid load control sample, Sultana H5 (Fig. 3.6a). GYSV1 was detected in a lower limit of 16 pg total RNA by RT-PCR in

Fig. 3.6: Comparison of the sensitivity of detection of GYSV1 in Sultana H5 by RT-PCR and dot blot hybridization. DNase-treated Sultana H5 nucleic acid extracts (4 µg) were diluted ten-fold serially in 0.1 mM EDTA containing 10 ng/µl BSA (Bresatec, Australia). Aliquots of each dilution (4 µl) were used for RT-PCR and dot blot hybridization. **(a)** RT-PCR assay for GYSV1 using the c4 h4 primer pair and and cycle parameters in Table 3.1. In **lanes 1-10**, were 160 ng, 16ng, 1.6 ng, 160 pg, 16 pg, 1.6 pg, 160 fg, 16 fg, 1.6 fg and 0.16 fg template respectively. In **lane 11**, was the negative control without template. PCR products (in 5 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments. **(b)** Dot-blot hybridization using ^{32}P-labelled GYSV1 riboprobe for viroid detection. **Samples 1-10** contained 4 µg, 400 ng, 40 ng, 4 ng, 400 pg, 40 pg, 4 pg, 400 fg, 40 fg and 4 fg template respectively.

Fig. 3.7: Combined RT-PCR assay on a Shiraz vine.

Nucleic acid extracts of Shiraz BVRC30/V2 (non-DNase-treated) were diluted with 0.1 mM EDTA containing 10 ng/µl BSA to the following concentrations in **lanes 1- 9**: 150 ng, 50 ng, 25 ng, 2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, 25 fg and 2.5 fg respectively. Assay for HSV was carried out using c1h1 HSV and cycle parameters shown in Table 3.1. PCR products (in 8 µl) were separated on a 1.5% agarose gel. The expected size of the PCR product was 297 bp. The DNA size markers were pUC 19/*Hpa*II fragments.

(a)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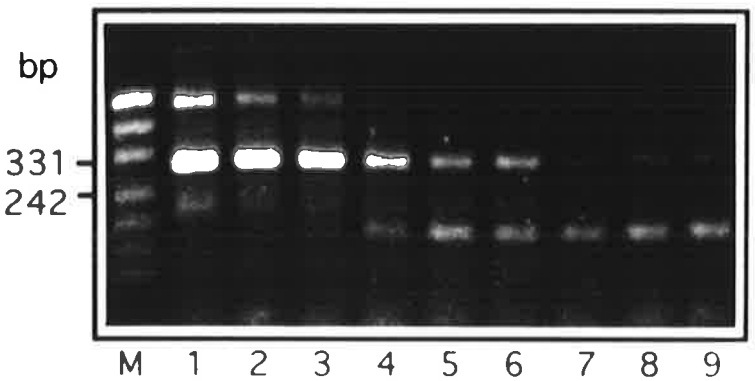
M 1 2 3 4 5 6 7 8 9 10 11 M

(b)


1 2 3 4 5 6 7 8 9 10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M 1 2 3 4 5 6 7 8 9

contrast to a lower limit of 400 ng total RNA by dot blot hybridization using ^{32}P-labelled riboprobes (Fig. 3.6b). Thus, a 25,000-fold increase in the sensitivity of viroid detection was obtained by RT-PCR.

RT-PCR assay was carried out as described above on 10-fold serial dilutions of a Ramsey rootstock, known to have a high GYSV1 load from dot blot hybridization analysis and graft indexing data previously obtained by Cirami et al. (1988). This Ramsey had a class 4 hybridization dot intensity (Table 4.1) compared to a class 1 dot intensity for Sultana H5 (Table 3.2). Amplification by PCR using the c4h4 GYSV1 primer pair and the cycle parameters in Table 3.1 produced, contrary to expectations, an extremely low yield of the expected PCR product. The low yield was maintained in serial 10-fold dilutions of the extract, indicating that inhibition was not likely to be the cause of non-amplification as had been suggested by Rezaian et al. (1992). This was confirmed by the high yield obtained when 1 µl of a 10,000-fold dilution of the Sultana H5 PCR product was included as template in the PCR reaction mixture for the Ramsey rootstock.

To test whether sequence variation in the GYSV1 population of the Ramsey rootstock was responsible for the low level of amplification, the number of PCR cycles was increased from 25 to 30 and 35. Under these conditions, an increasing yield of the PCR product was obtained (data not shown), confirming a low concentration of amplifiable templates or a low efficiency of amplification as the cause for the low yield observed previously.

3.3.2.4. Combined RT-PCR (cRT-PCR)

In an attempt to reduce the number of processing steps for the purpose of routine indexing, cRT-PCR, based on the work done by Sellner et al. (1992), was carried out on non-DNase-treated nucleic acid extracts in single reaction tubes (0.5 ml) in an integrated thermal cycling program. Fig. 3.7 shows the results obtained on assaying serial dilutions of a Shiraz BVRC30/2 vine for HSV using c1h1 HSV primers (Table 3.1). A high yield of the expected PCR product (297 bp) was obtained together with minor bands of non-specific products. HSV was clearly detected in 25 pg of total nucleic acid (lane 6), giving

a sensitivity equivalent to that obtained by the optimised RT-PCR protocol by fast cycle times (See 3.3.2.3). Hence, this cRT-PCR protocol, which eliminates two time consuming steps, the DNase treatment and the separate reverse transcription reaction, in exchange for a slightly reduced specificity, may have an application for large scale screening. However, it is applicable as a fast screening procedure only to high viroid load samples since the expected PCR product is no longer the major product in low viroid samples (Fig. 3.7, lanes 5-9) and confirmation with further assays may be required.

3.3.2.5. cRT-PCR by fast cycle times

The cRT-PCR was adapted for fast cycle times for amplification in capillary tips. Amplification was observed only in the presence of the reaction buffer for fast cycle times. The cRT-PCR assay by fast cycle times of Sultana H5, using the primer pair, c3h2 HSV (Table 3.1), gave large bands of the expected product together with a single high molecular weight non-specific product similar to that obtained in standard cRT-PCR (data not shown). The specificity of cRT-PCR by fast cycle times was increased with the use of DNase-treated samples, as expected. However, the best yield, comparable to that obtained by the optimized protocol, was obtained with only 40 ng template; with more template, the yield remained constant and a high background smear was observed. Of the four protocols studied, the optimised protocol gave the highest specificity and was the protocol of choice for low titre templates. The usefulness of cRT-PCR by fast cycle times was in the further reduction of the time required for preliminary screening procedures as compared to cRT-PCR so that the indexing of a given RNA sample could be completed in less than 3 h.

3.3.2.6. Evaluation of other reagents and methods reported to increase the specificity of PCR

In earlier attempts in this study to improve the specificity of amplification by PCR, organic solvents such as formamide, glycerol, NP-40, DMSO, Tween-20 (Varadaraj and Skinner, 1994) and tetramethylammonium chloride (Chevet et al., 1995) were used

without success (data not shown). The Touchdown PCR technique (Don et al., 1991) did not produce the high specificity required. However, a minor improvement in the specificity of amplification of low titre templates was observed with TaqStart™ Antibody (Kellogg et al., 1994), without an increase in the product yield (data not shown). The nested priming effect (Haff, 1994) was used to enhance the specificity of amplification of GYSV1 in the 10 grape varieties (See below), using c1GYSV1 reverse primer for reverse transcription and c4h4 GYSV1 primers for PCR. The yield obtained was at least 1000-fold reduced in comparison to using c4 GYSV1 primer in reverse transcription (data not shown).

Thus, the careful selection of primers as well as the optimization of the parameters of the PCR process, which was an important feature of this new RT-PCR assay, proved to be the most effective approach to increase specificity.

3.3.3. Assay of the five grapevine viroids in 10 grapevine varieties

3.3.3.1. Dot-blot hybridization assay

Dot-blot hybridization assays were carried out only for GYSV1, HSV, AGV and CEV using ^{32}P-labelled riboprobes. An assay was not carried out for GYSV2 because cross-hybridization between GYSV1 and GYSV2 had been reported (Koltunow et al., 1989) and the author believed it was not possible to discriminate between these two viroids. Hence, dot blot hybridization assay was not carried out for GYSV2 for any test sample throughout this thesis. However, at a very late stage near the end of the experimental part of this thesis, it was found that under the conditions used, the probe for each viroid was in fact highly specific. Although this technique was known to be less sensitive than RT-PCR, it was used to confirm the positive results obtained in higher viroid load samples by RT-PCR, because of the risk of false positives that could arise with PCR-based techniques.

Two steps were included in the dot blot hybridization procedure because of their positive effects: (1) the preliminary washing of the membranes before prehybridization (2) the use of fresh hybridization buffer before the addition of probes.

The preliminary washing (Hanold et al., 1993) removed RNAs not properly bound to the membrane,which otherwise came off during prehybribridization and re-bound to the labelled probe causing a number of non-specific dark signals on the final autoradiographs. This released RNA, apart from diluting the effective concentration of the labelled probe, was also reported to hybridise to the filterbound RNA because of the self complementary nature of viroid sequences, lowering the signal obtainable (Hutchins et al., 1985; Branch and Robertson, 1984). Problems arising from this excess RNA were also observed by Sheldon (1992). The use of fresh buffer after the prewash allowed a clear background to be obtained and signals of low intensity to be picked up.

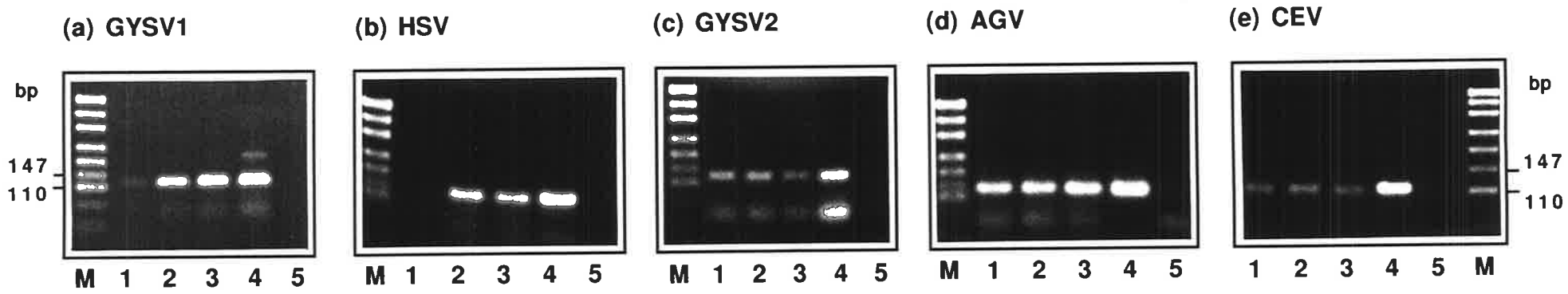
Dot blot hybridization using ^{32}P-labelled viroprobes detected GYSV1, HSV and AGV in all 10 varieties with the latter viroid in much lower titre, while CEV was beyond the limit of detection (Table 3.2). GYSV1 and AGV were also detected in the Emperor seedlings, RTG and Vfr. The presence of AGV could not be shown earlier in this study using ^{32}P-labelled cDNA probes generated by random priming.

From the dot blot hybridization results (Table 3.2), a viroid profile was apparent for each vine assayed based on the intensity of the hybridization dots obtained, using ^{32}P-labelled riboprobes specific for each viroid and assayed under the conditions defined (See 3.2.12). Such viroid profiles enabled a direct comparison of the viroid content between samples assayed in the same hybridization batch.

3.3.3.2. RT-PCR assay

The 10 grapevine varieties were indexed by RT-PCR for the five grapevine viroids using the partial length primers listed in Table 3.1. Fig. 3.8 shows the indexing by RT-PCR of two varieties, Shiraz and Sultana, with an *in vitro* germinated Emperor seedling, RTG, as a putative negative control. However, this seedling was found to be positive for GYSV1, GYSV2, AGV and CEV and negative for HSV (Fig. 3.8 a-e, lanes 1), contrary to expectations and to previous reports on vine seedlings as viroid-free tissue (See 1.7.6). The results were confirmed on a second Emperor seedling, Vfr. The Shiraz and the Sultana vines assayed positive for all five viroids (Fig. 3.8 a-e, lanes 2 and 3).

Fig. 3.8: Assay of the five grapevine viroids in two grape varieties and one seedling by RT-PCR. The samples in (**a**)-(**e**) were in **lanes 1-3**, seedling Emperor RTG, Shiraz BVRC 30/BV8 and Sultana H5 vines respectively. In **lanes 4**, viroid-specific PCR products diluted 5000-fold were used as control templates. In **lanes 5**, were the negative controls without templates. Amplification was carried out using the cycle parameters given in Table 3.1 and the following primers: in (**a**) c4h4 GYSV1 (**b**) c3h2 HSV (**c**) c1h3 GYSV2 (**d**) c2h2 AGV (**e**) c1h3 CEV. The expected sizes of the PCR products were for GYSV1, 120 bp; HSV, 138 bp; GYSV2, 123 bp; AGV, 130 bp, CEV, 115 bp. PCR products (in 4 µl) were separated on a 2% agarose gel for (**a**)-(**d**) and on 4% agarose gel for (**e**). The DNA size markers were pUC 19/*Hpa*II fragments.


(a) GYSV1
(b) HSV
(c) GYSV2
(d) AGV
(e) CEV
bp
147
110
M 1 2 3 4 5
M 1 2 3 4 5
M 1 2 3 4 5
M 1 2 3 4 5
1 2 3 4 5 M
bp
147
110

The results of the RT-PCR analysis of all 10 grape varieties are shown in Table 3.2. A good correlation was observed between the results obtained by dot blot hybridization and RT-PCR for GYSV1, HSV and AGV, confirmed by the negative results obtained for HSV in the two seedlings by both methods. Additional experiments were carried out to confirm the RT-PCR results not supported by dot blot hybridization analysis. For CEV, the results were confirmed by repeating the RT-PCR indexing for all 10 samples. The presence of GYSV2 in all 10 samples was confirmed by indexing with two primer pairs, c1h2 and c2h1 GYSV2 (Table 3.1), and additionally with the c1h3 primer pair for the RTG seedling, Shiraz and Sultana (Fig. 3.8).

Hence, the viroid profiles of the 10 grapevine varieties obtained by RT-PCR, as determined by the presence or absence of a viroid specific band after amplification for each viroid, differed from those obtained by dot blot hybridization assay. However, this was expected in view of the difference in sensitivity between the two techniques.

3.4. Discussion

3.4.1. Influence of RNA extraction on viroid detection

A high sensitivity RT-PCR assay for grapevine viroids, which uses an extraction method that yielded high quality RNA from grapevines, is reported here. A number of other extraction methods (Palukaitis and Symons, 1980; McInnes et al., 1989; Doyle and Doyle, 1990; Collins and Symons; 1992, Levi et al., 1992) had been used previously without success. The high yield and quality of RNA obtained by this protocol could perhaps be related to the absence of phenol, commonly used in other extraction buffers. Phenol was reported to interfere severely with RNA extraction in grapevines (Flores et al., 1985; Semancik et al., 1987), causing huge losses, particularly in the yield of the higher molecular weight RNA (Rezaian and Krake,1987), when present in the initial extraction buffer. It was also reported to affect the quality of RNA as a result of the reaction of phenol with vine polyphenolics to form compounds which co-purified with RNA and resisted subsequent attempts at elimination.(Loomis, 1974; Van Driessche et al., 1984).

Table 3.2: Dot blot hybridization and RT-PCR assay of the five grapevine viroids in 10 grapevine varieties and a seedling

Grape varieties	GYSV1		GYSV2		HSV		AGV		CEV	
	DB	RT-PCR	DB	RT-PCR	DB	RT-PCR	DB	RT-PCR	DB	RT-PCR
Emperor RTG	1	+	nt	+	0	-	1	+	0	+
Riesling	2.5	+	nt	+	3	+	1	+	0	+
Chardonnay	2	+	nt	+	3	+	1	+	0	+
Sauvignon Blanc	2	+	nt	+	2.5	+	1	+	0	+
Grenache	1.5	+	nt	+	2	+	1	+	0	+
Colombard	1	+	nt	+	3	+	1	+	0	+
Pinot noir	4	+	nt	+	3.5	+	1	+	0	+
Mataro	1	+	nt	+	1	+	1	+	0	+
Pedro Ximenes	1	+	nt	+	1	+	1	+	0	+
Shiraz	1	+	nt	+	3.5	+	1	+	0	+
Sultana	1	+	nt	+	3.5	+	1	+	0	+

+, -, indicates the presence or absence of a viroid specific band respectively.

nt: not tested

DB: Dot blot hybridization assay.

An arbitrary scale was assigned to the dots to reflect their intensity. Results rated from 0 (negative result) to class 5 (highest level of infection). Ramsey vine A1S10 from PISA was used as reference sample for class 5 dot.

Primer pairs used for RT-PCR analysis were: c4h4 GYSV1, c2h2AGV, c1h3 CEV, c1h1 HSV and for GYSV2, the two primer pairs c2h1GYSV2 and c1h2 GYSV2 (See Table 3.1). The results obtained above on RTG, Shiraz and Sultana H5 vines were confirmed additionally with c3h2 HSV and c1h3 GYSV2 primer pairs (Fig. 3.8).

RNA extracted by the protocol described here was found to be suitable for RT-PCR analysis without the requirement for additional purification steps, e.g., CF11 cellulose chromatography and the re-precipitation of viroid nucleic acids with 2 M LiCl (Flores et al., 1985, Duran-Vila et al., 1988; Semancik et al., 1987; Szychowski et al., 1988; Rezaian et al., 1992), commonly used for viroid purification and which may cause losses of viroid RNA. For example, the CF11 cellulose column, which was originally developed for purifying double-stranded RNA (Franklin, 1966) might not be as efficient for binding viroids (Semancik, 1986) because of the presence of single-stranded loops in the structure of viroids. Earlier in this study, the efficiency of amplification of GYSV1 by RT-PCR was found to be decreased after prolonged incubation of RNA extracts with 2 M LiCl (data not shown). In one instance, HSV was detected exclusively in the pellet rather than in the supernatant after overnight treatment with 2 M LiCl. Many workers have previously reported a requirement for the dilution of RNA extracts before RT-PCR analysis to reduce or abolish interference from inhibitors (Borja and Ponz, 1992; Minafra and Hadidi, 1994; Barbarossa et al., 1994; Hu et al., 1995). With the present extraction protocol, the best RT-PCR signal was obtained with undiluted RNA extracts.

3.4.2. A new viroid profile for grapevines by RT-PCR assay with all five grapevine viroids

The development of this RT-PCR protocol with fast cycle times enabled the reliable, sensitive and non-radioactive detection of all five grapevine viroids directly in extracts of vine tissue. The simultaneous infection by all five grapevine viroids in the 10 grape varieties tested, as indexed by RT-PCR (Table 3.2), was in contrast to the previously reported infection by at most three viroids at a time in most vines using a less sensitive detection method, sPAGE followed by silver staining (Szychowski, et al., 1991; Semancik et al., 1992). However, the presence of four viroids, GYSV1, GYSV2, HSV and AGV, had been previously reported in two vines, the Californian Thomson Seedless and the Australian Sultana D1H, using ^{32}P-labelled cDNA transcripts from M13 viroid clones as probes (Rezaian et al., 1992). Low-titre CEV was detected for the first

time directly in grapevine tissue without passage through alternative hosts (Rezaian et al., 1988). The presence of viroids in the Emperor seedlings provided the first report on the transmission of viroids via seeds in grapevines. GYSV1, GYSV2, AGV and CEV, but not HSV, were detected in two Emperor seedlings. The general applicability of viroid transmission via seeds needs to be confirmed in other grape varieties.

3.4.3. Sequence variation in the amplification of viroids by RT-PCR

The present study revealed sequence variation as a important factor to be considered in the indexing of viroids by RT-PCR. Amplification of viroids, compared to other templates, was far more complex because of the existence of viroids as quasi-species (Eigen et al., 1988) i.e., as a population of sequence variants all related to a consensus sequence and differing from it by one or more base changes presumably as a result of the high error rates of RNA polymerases, rather than as a population of identical sequences. In the case of GYSV1, the existence of a large number of main sequence variants (Rigden and Rezaian, 1993; Polivka et al., 1996) further complicated the situation. Hence, GYSV1, although shown to be present in the Ramsey rootstock in high titre by dot blot hybridization assay, could not be amplified efficiently using the same primer pair and cycle parameters as used for other vines.

Because GYSV1 variants, with base changes in the inverted repeats bordering the CCR, were reported by Polivka et al. (1996) a search for sequence variants of GYSV1 was made on the EMBL nucleotide sequence database to exclude nucleotide changes in the c4 and h4 primer region as a possible reason for the failure to amplify this viroid in the Ramsey rootstock. No sequence variation was found in the region covered by both primers. This suggested that the Ramsey rootstock most likely had an interesting population of unknown sequence variants with high replication efficiency as well as a high level of pathogenicity.

The difference in sensitivity of viroid detection obtained between Sultana H5 and the Ramsey rootstock by RT-PCR indicated sequence variation as a factor to be considered in viroid indexing by RT-PCR. Hence, the 25,000-fold increase in the

sensitivity obtained above with Sultana H5 only indicated the possibility of detection of low level GYSV1 in a vine where the GYSV1 population was amplifiable by the primer pairs used and could not be used without qualification as a general reference for amplification in every vine assayed.

Less obvious influences of sequence variation found in the course of this study include the reduced yield obtained when nested priming was used to improve the specificity of PCR (See 3.3.2.6). The explanation for this observation was obtained in the work of Polivka et al. (1996). The high level of sequence variation they found in the region covered by the GYSV1 c1 primer led to their proposal for most of this region, including 17 of the 21 nucleotides of the c1 primer, originally assigned to the T1 domain by Rigden and Rezaian (1993), to be included instead in the V domain of GYSV1.

3.4.4. Contamination in the routine use of RT-PCR assay

Although this newly-developed RT-PCR protocol has the sensitivity and reproducibility required for routine screening, the major problem in its application on a large scale will be, as for all RT-PCR based techniques, the control of contamination. Even with all the precautions taken (See 3.2.6.5), at times the indexing had to be stopped for several weeks before it could be successfully resumed. Carryover contamination, as a result of the accumulation of PCR products from previously amplified material, especially in air-conditioned laboratories, remains the greatest technical challenge to the regular use of RT-PCR in indexing. Although a number of chemical decontamination systems have been described, a recent review by Niederhauser et al. (1994) concluded that the physical containment of contamination, as described by Kwok and Higuchi (1989), was the most efficient means to control this problem. With the risk of contamination increasing with the sensitivity of the amplification, the role of negative control samples assumes a greater importance. For large scale indexing, a greater number of samples without templates, at least one per 10 samples, may need to be used for the negative control to achieve its aim.

Chapter Four

Viroids in Meristem-regenerated Vines

4.1. Introduction

In vitro meristem culture techniques have been used as an approach to produce pathogen-free vines (Hewitt, 1990). Of the two culture techniques used, shoot apical meristem culture (SAMC) has been reported to produce viroid-free vines (Duran-Vila et al., 1988) while fragmented shoot apex culture (FSAC) (Barlass and Skene, 1978) did not eliminate viroids (Habili et al., 1992). These two techniques differ in the sizes of explants used: 1.0 mm long apices with more than two leaf primordia and cut into 20 pieces were used for FSAC; 0.1- 0.2 mm whole meristem tips with a maximum of two leaf primordia were used for SAMC (Salazar et al., 1985). Further, while FSAC-derived vines regenerate through adventitious bud formation from leaf primordial fragments, SAMC-derived vines regenerate through the expansion and proliferation of the whole apical meristem.

Of the five grapevine viroids (See 1.7), GYSV1 and HSV were first reported to be successfully eliminated from *Vitis vinifera* L. Cabernet Sauvignon by SAMC (Duran-Vila et al., 1988). A comparison of the viroid profiles of the stock vines and the SAMC-derived vines after sPAGE and silver staining (See 1.8.3) indicated that viroids had been eliminated by meristem culture. Subsequently, putative viroid-free clones were produced from 32 grapevine cultivars by this technique, although difficulties were reported with the elimination of GYSV1 and GYSV2 from certain cultivars, among which were Cabernet Franc, Merlot, Chardonnay and the rootstock 41-B (Juarez et al., 1990).

From a survey of previous reports on viroid-free plants (Table 4.1), a preliminary cold treatment of the stock plants prior to meristem culture appeared to be favourable for viroid elimination in certain plant species (Lizárraga et al., 1980; Paduch-Cichal and Kryczyñski, 1987). Limitations to viroid detection due to the assay techniques were reported by many workers, with samples, initially testing negative, assaying positive at a later stage probably because a build-up of viroid level was required before detection by the assay technique used (Lizárraga et al., 1980; Paduch-Cichal and Kryczyñski, 1987). More sensitive diagnostic methods detected viroids in vines previously indexed as viroid-free. Thus, vines regenerated by FSAC, previously reported to be healthy by graft

Table 4.1: Viroid-free plants regenerated by meristem culture

Host / viroid	Stock plant		Meristem size(mm)	No. meristems		Assay method	References
	treatment	months		survived excised	viroid-free		
Potato / PSTV	5-6°C, 500 lx	6	dome+1p	13/48	7	Bio/PAGE*	Lizárraga et al., 1980
Potato / PSTV	25°C, 1500 lx	2	"	16/48	0	"	"
Potato tubers / PSTV	8°C, 5000 lx	4	"	17/48	5	"	"
Potato plants / PSTV	greenhouse	4	"	16/48	0	"	"
Hops / HSV	greenhouse	ns	0.2-0.4	15/51	3	Ultrastructure	Momma and Takahashi, 1983
Hops / HSV	10°C, 5400 lx	1-4	0.2-0.3	10/103	1	Ultrastructure	Momma and Takahashi, 1983
Tomato / CEV	greenhouse	ns	0.2-0.4	23/67	6	Bio/PAGE*	Duran-Vila et al., 1986
Grapevine / GYSV1, HSV	greenhouse	ns	0.1-0.2	17/17	17	"	Duran-Vila et al., 1988
Potato / s-PSTV	6-7°C, 5000 lx	6	dome+2p	22/38	9	"	Paduch-Cichal and Kryczyñski, 1987
Potato / m-PSTV	6-7°C, 5000 lx	6	dome+2p	18/40	9	"	"
Chrysanthemum / ChCMV	"	6	"	37/57	26	"	"
Chrysanthemum / CSV	"	6	"	27/75	5	"	"
Chrysanthemum / HSV	"	6	"	10/30	8	"	"
Hops / HLV	10-25°C	8-28d	0.5-10	132/ns	0	^{32}P-riboprobe	Morton et al., 1993
Hops / HLV	ns	ns	ns	7/ns	2	RT-PCR	Hataya et al., 1992

p, leaf primordium; ns, not specified; s, severe; m, mild.

*, Bioassay and 5% native PAGE followed by overnight staining with 0.1% toluidine blue (Morris and Smith, 1977).

indexing, were later found to be infected, by hybridization analysis using ^{32}P-labelled cDNA probes (Koltunow et al., 1989; Habili et al., 1992). From more recent work using more sensitive viroid detection techniques, it is increasingly evident that viroid-free plants are difficult to produce by meristem culture. For example, Morton et al. (1993) detected hop latent viroid (HLV) in all SAMC-derived hops by dot-blot hybridization using ^{32}P-labelled riboprobes, while Hataya et al. (1992) detected HLV by RT-PCR assay in regenerated hops previously reported to be negative by dot-blot hybridization assay. The work of Hataya et al. (1992) was of particular interest because it showed, using three assay methods, the existence of a range of viroid concentrations among the regenerated vines, from those detectable by 2-D PAGE (See 1.8.3) to those no longer detectable by RT-PCR. It also demonstrated the critical influence of the assay technique on the result obtained, with the number of viroid-free plants decreasing as the sensitivity of the assay technique increased.

The objective of the work presented in this chapter was to evaluate the viroid status in vines produced by FSAC and SAMC by a combination of dot blot hybridization analysis using ^{32}P-labelled riboprobes and RT-PCR.

4.2. Materials and Methods

4.2.1. Acknowledgements

The work carried out in this chapter has been made possible only through the availability of SAMC-derived vines produced in 1990-1991 by the Victorian Plant Research Institute, Burnley, Victoria, and the FSAC-derived vines produced in 1991 by Primary Industries South Australia (PISA) .Although the vines produced at Burnley were initially meant to be produced by the proven technique of FSAC for virus elimination, they were in fact produced by SAMC. According to Mr Rob Bennett, who was involved in producing these vines, all the regenerated vines were derived from the apical dome and a maximum of two leaf primordia. SAMC and FSAC-derived vine samples were obtained from PISA with the assistance of Dr Richard Hamilton. Samples from SAMC-derived vines, growing under field conditions, were obtained from VAMVIA, Irymple, Victoria, kindly provided by Mr Graham Fletcher. Vine samples from Irymple were collected by Mr Robert Bennett and indexed by Ms Sonia Dayan.

4.2.2. Sources of plant material

Nineteen FSAC and 14 SAMC-derived vines were obtained from PISA, produced from two Cabernet Sauvignon stock vines, CS LC30 and CS 125, and a Ramsey rootstock (See Table 4.2). SAMC-derived vines were produced by the method of Duran-Vila et al. (1988). FSAC-derived vines were produced according to the method of Barlass and Skene (1978). Regenerated vines were obtained either as (1) plantlets maintained *in vitro* or as (2) vines planted in the shadehouse since 1991. The *in vitro* vine samples were obtained in 1993-1994 and maintained by subculturing on Murashige and Skoog (MS) culture medium (Murashige and Skoog, 1962) supplemented with the plant growth regulator 6-benzyladenine (BA) at a concentration of 5 μM, every 8-10 weeks.

Fifty SAMC-derived vines were obtained from VAMVIA, with at least two regenerated vines per stock plant. The stock vines used were: nine rootstocks, Ramsey, Richter 99, Richter 110, Ruggeri 140, Kober 5BB, Dog Ridge, Paulsen 1103, Teleki 5A, Schwartzmann and 12 wine-grape varieties, Gamay, CS G9V3, CS 125, Chardonnay G9V7, Chardonnay 110V5, Pinot Noir D2V5, Ruby Cabernet E5V4, Russian Seedless, Cabernet Franc C7V15, Graciano WA6V6, Sultana H5 and Riesling F8V13.

The control vine samples used in Northern hybridization analysis were Sultana H5 (See 3.2.1), BVRC30/BV8 (See 5.2.1) and its micropropagated progeny vine, BVRC30/BV8/15 (See Table 5.1), which had been subcultured for 15 cycles.

4.2.3. RNA extraction

RNA extraction was carried out as described previously (See 3.2.3). RNA was extracted directly from the tissue-cultured leaves of micropropagated vines. The average yield of RNA from these leaves was much lower than that from shadehouse or field-grown leaves. An average yield of 83.6 μg/g fresh weight of leaf was obtained from the extraction of 33 micropropagated vine samples, equivalent to 26.5% of the yield from field-grown leaves.

4.2.4. Viroid assays

Dot blot hybridization analysis was carried out on vine RNA extracts using ^{32}P-labelled GYSV1, HSV, AGV and CEV riboprobes prepared as described previously (See 3.2.11). Nucleic acid extracts for each sample (4 µg per dot) were prepared in a bulk mixture and applied simultaneously to each of four replicate membranes as described previously (See 3.2.10).

The samples from PISA and VAMVIA were assayed in a single hybridization assay (See 3.2.12) together with samples from the following experiments to enable a comparative assessment of the viroid concentrations: (1) 10 grape varieties (Chapter 3); (2) stock and micropropagated vines (Chapter 5); (3) 11 seedlings (Chapter 6); and (4) the cold-treated stock vines (Chapter 7). Negative controls for dot blot hybridization assays were as described in section 3.2.10.

RT-PCR assays were carried out by the optimised protocol for fast cycle times (See 3.2.5 and 3.2.6.2) on selected samples found to be negative by dot blot analysis. The primer pairs used were c4h4 GYSV1 and c3h2 HSV (Chapter 3, Table 3.1).

4.2.5. Northern hybridization analysis

Samples of total nucleic acid extracts (16 µg, non-DNase treated) were separated in a 5% polyacrylamide-7M urea gel (See 2.2.2.2) and transferred to Hybond N+ membranes (See 2.2.6.2). Hybridization analysis was carried out on the membranes using ^{32}P-labelled GYSV1 full-length riboprobes as described previously (See 3.2.11-3.2.12).

4.3. Results

4.3.1. Viroids in meristem-regenerated vines from three stock vines

Dot blot hybridization assays of the four SAMC-derived CS vines from WaggaWagga (See 3.3.2) had previously indicated that viroids were readily detectable in vines regenerated by this technique. The varying intensity of the hybridization dots

enabled a quantitative estimation of the viroid concentration. Hence, dot blot hybridization assays were carried out on all 33 vines from PISA, regenerated both by SAMC and FSAC; RT-PCR was then carried out only on selected samples found to be viroid-negative by the hybridization assay.

4.3.1.1. SAMC-derived vines

The results obtained on 14 SAMC-derived vines, comprising seven CS LC30, five Ramsey and two CS 125, are shown in Fig. 4.1. The results, based on the intensity of the hybridization dots on a scale from class 0, negative viroid content to class 5, the highest level of infection observed, are summarized in Table 4.2. GYSV1 and AGV were present in all 14 vines. HSV was not detected in any CSLC30 regenerant. However, HSV was found in Ramsey R1 and in two CS125 regenerants S1 and S2.

Vines regenerated from the low titre CS LC30 and CS 125 stock vines maintained the same low level of GYSV1 as initially present in their stock vine. Most regenerants from the Ramsey rootstock with a high initial GYSV1 content maintained the same or slightly reduced levels of GYSV1, except for Ramsey R5, which showed a considerably reduced level of viroids. Two regenerants, CS LC30 L6 and Ramsey R1, showed an increase in GYSV1 content compared to the stock vines from which they were derived. Contamination by external sources as a possible cause for this increased titre was ruled out in the case of L6, because this vine had been maintained *in vitro* since SAMC.

4.3.1.2. FSAC-derived vines

The 19 FSAC-derived vines, comprising four Ramsey and 15 CS 125 vines, gave results essentially similar to those obtained with the SAMC-derived vines. GYSV1 and AGV were found in all the FSAC-derived samples while HSV was detected in only seven of these vines (Table 4.2).

All the CS 125 regenerants showed a level of GYSV1 and AGV comparable to their stock vine, except for S9,S10, S11 and S12, which were slightly reduced in AGV content. The maintenance of the same level of GYSV1 titre in all CS 125 vines, whether

Fig. 4.1: Viroid profiles in PISA vines regenerated by SAMC and FSAC as determined by dot blot hybridization

The stock vine samples and the SAMC and FSAC-derived vine samples used in the assays were as follows:

Row 1, CS LC30 stock vine and seven SAMC-derived vines, L1-L7.

Row 2, Ramsey rootstock, five SAMC-derived vines, R1-R5, and four FSAC-derived vines, R6-R9.

Row 3, CS 125 stock vine, two SAMC-derived vines, S1-S2, and five FSAC-derived vines, S3-S17.

Row 4, 10 CS 125 FSAC-derived vines, S8-S17.

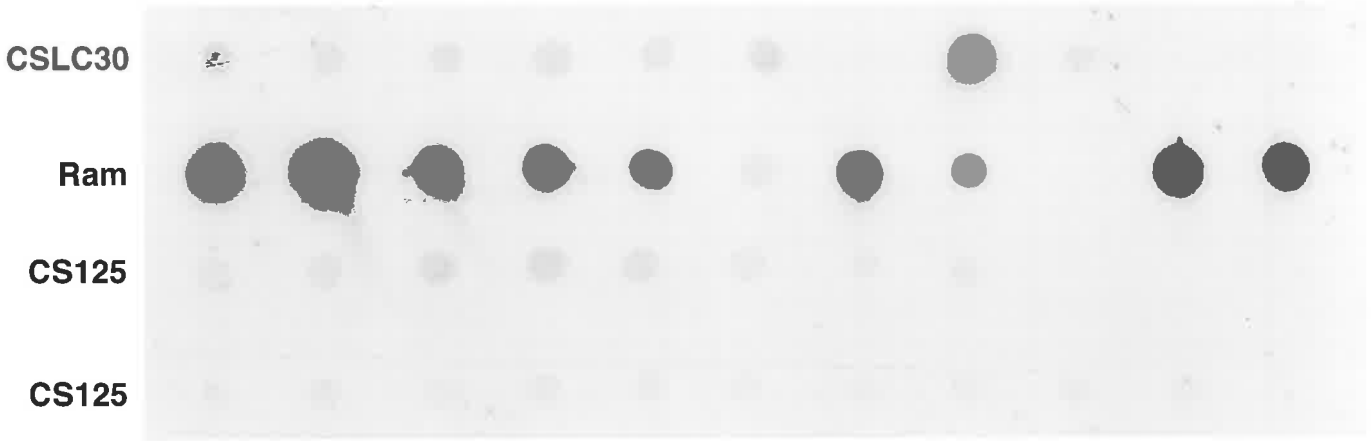
1	CS LC30 stock	L1	L2	L3	L4	L5		L6	L7		
2	Ramsey stock	R1	R2	R3	R4	R5	R6*	R7*		R8*	R9*
3	CS 125 stock	S1	S2	S3*	S4*	S5*	S6*	S7*			
4	S8*	S9*	S10*	S11*	S12*	S13*	S14*	S15*	S16*	S17*	

*Samples regenerated by FSAC; other vines were regenerated by SAMC.

Samples underlined were from *in vitro* grown vines. All other samples were from vines grown in the SARDI shade house.

Only results for GYSV1, HSV and AGV are shown here since CEV was not detected in any sample. The negative control vines for GYSV1 and AGV were as described in Chapter 3, Table 3.2. The negative control vines for HSV were the two Emperor seedlings, RTG and Vfr.

GYSV 1



HSV

AGV

Table 4.2: Viroid profiles in PISA vines regenerated by SAMC and FSAC as determined by dot blot hybridization

Stock vines & derivatives	Regenerant vines SAMC	FSAC	GYSV1	HSV	AGV	CEV
Stock CS LC30			1.5	1	1	0
L1	R2 A2 S 26		1	0	1	0
L2	R2 A2 S 1		1	0	1	0
L3	R2 A2 S 5		1	0	1	0
L4	R2 A6 S 3		1	0	1	0
L5	R2 A2 S 16		1	0	1	0
L6	R2 A5 S12		**4**	0	0.5	0
L7	R2 A6 S3		1	0	0.5	0
Stock Ramsey			4	2	2	0
R1	A1 S10		**5**	1	1	0
R2	R2 A5 S6		3.5	0	1	0
R3	R2 A5 S15		3.5	0	1	0
R4	R2 A5 S12		3	0	1	0
R5	R2 A1 S25		1	0	1	0
R6		M30 F1D	3.5	0	1	0
R7		M30 F1C	2	0	1	0
R8		M1F2A	4	**2.5**	**2.5**	0
R9		M30 F1C	4	0	0.5	0
Stock CS 125			1	1	1	0
S1	R2 A7 S1		1	1	1	0
S2	A2		1	1	1	0
S3		E5 F2B	1	1	1	0
S4		M4 F2C	1	0	1	0
S5		M6 F1D	1	0	1	0
S6		E2 F2 B	1	1	1	0
S7		E2 F2 D	1	1	1	0
S8		M5 F1 A	1	0	1	0
S9		M7 F1 B	1	0	0.5	0
S10		M4 F2 C	1	0	0.5	0
S11		M7 F1 A	1	0	0.5	0
S12		E2 F1 A	1	1	0.5	0
S13		M6 F1 D	1	0	1	0
S14		M1 F1 A	1	0	1	0
S15		M1 F1 B	1	0	1	0
S16		M4 F3 A	1	0	1	0
S17		E5 F2B	1	1	1	0

Samples underlined were from vines maintained *in vitro* after meristem culture. All other samples were from shadehouse-grown vines.
Numbers in bold indicate increases in titre relative to the stock vine.
Hybridization dots were rated from 0 to class 5 as described in Chapter 3, Table 3.2.

grown *in vitro* or in the shadehouse, implied that viroid metabolism was not significantly different in vines grown under the two conditions. It also suggested that extraction from *in vitro* leaves, although giving a quarter of the yield obtained from shadehouse-grown leaves, did not influence the viroid assay to a major extent.

CS 125 samples, S4 and S5, extracted from shadehouse-grown leaves, showed GYSV1 and AGV profiles (See 3.3.3.1) similar to those obtained in samples S8 and S13-S17 extracted from *in vitro* cultured leaves (Table 4.2). Further, samples S3 and S17, which were clones derived from the same regenerant vine but maintained in two different conditions, in the shadehouse and *in vitro* respectively, had identical viroid profiles.

However, the absence of an observable effect on the viroid content in vines after micropropagation for 4 years was most unusual because micropropagation (Chapter 5) was generally observed to increase GYSV1 and HSV titres to high levels in most micropropagated vine samples (See Table 5.1). The GYSV1 content in the SAMC-derived vines, that remained stable at low level, could be attributed either to the existence of only low titre GYSV1 variants in the initial viroid population of the stock vines or to the existence of a GYSV1 population, as a consequence of meristem culture, which could no longer be induced to high titres. A definite conclusion could not be reached in the case of CS 125 vines because the effect of micropropagation on their stock vine was not known. However, in the case of CS LC30 vines, the high GYSV1 content observed in L6 (Table 4.2) suggested the presence of high titre GYSV1 variants in the stock vine, since infection by external sources was excluded by the maintenance of L6 *in vitro* after meristem culture. The maintenance of a low GYSV1 content in the *in vitro* -grown L7 (Table 4.2) suggested rather that GYSV1 variants, which replicated to high titres, had been eliminated by SAMC in this vine. The behaviour of HSV in the SAMC-derived vines under micropropagation conditions also suggested a selective elimination of high titre HSV variants as a result of SAMC.

Ramsey R8, an FSAC-derived vine maintained *in vitro*, showed an unusual viroid profile consisting of a very high level of GYSV1 and an increased level of HSV

compared to its stock vine, in contrast to the general trend towards the elimination or reduction of HSV in all other regenerants. An increase in AGV titre was observed at the same time in this vine.

4.3.1.3. Evaluation of SAMC and FSAC in relation to viroid elimination

The combined results obtained from FSAC and SAMC-derived vines, presented in Table 4.3, indicated that there was no major difference between these two culture techniques: neither technique eliminated viroids; further, both techniques produced vines with increased viroid titres (Table 4.2).

GYSV1 and AGV were present in all the regenerated vines tested. In vines derived from stock vines with low initial viroid load, both GYSV1 and AGV were maintained at low levels. In vines regenerated from stock vines with high initial GYSV1 load, e.g., Ramsey, only a slight reduction of GYSV1 content was observed by both methods, as found in SAMC-derived samples, R2-R5, and FSAC-derived samples, R6 and R7.

In contrast, HSV was detected in only nine of the 33 vines assayed, three SAMC-derived and six FSAC-derived vines, showing that HSV had been eliminated in most of the samples. Further analysis was carried out by RT-PCR on six of the HSV-negative samples: four SAMC-derived, L1, L2, L4 and S1, and the two FSAC-derived, S4 and S15 (see Table 4.2). HSV was clearly detected in all of these vines except L1 (data not shown). This latter vine was consistently negative in spite of further attempts to increase the sensitivity of the RT-PCR assay by increasing the number of PCR cycles and the amount of template. Hence, it was likely that in most regenerated vines, HSV was not eliminated but rather reduced to an extremely low level that was no longer detectable by ^{32}P-labelled riboprobes.

Table 4.3: Dot blot hybridization assay of viroids in vines regenerated by SAMC and FSAC from three stock vines

Cultivar	Culture technique	Regenerant vines tested	GYSV1		HSV		AGV		CEV	
			+	-	+	-	+	-	+	-
CS LC30	**SAMC**	7	7*	0	0	7	7	0	0	7
Ramsey	**SAMC**	5	5*	0	1	4	5	0	0	5
CS 125	**SAMC**	2	2	0	2	0	2	0	0	2
Ramsey	**FSAC**	4	4	0	1*	3	4	0	0	4
CS 125	**FSAC**	15	15	0	5	10	15	0	0	15
TOTAL	**SAMC FSAC**	**33**	**33**	**0**	**9**	**24**	**33**	**0**	**0**	**33**

* Included one vine with increased viroid load.

All 33 vine samples were obtained from PISA.

+, -, indicates the presence or absence of a hybridization dot respectively, using ^{32}P-labelled viroid specific riboprobes.

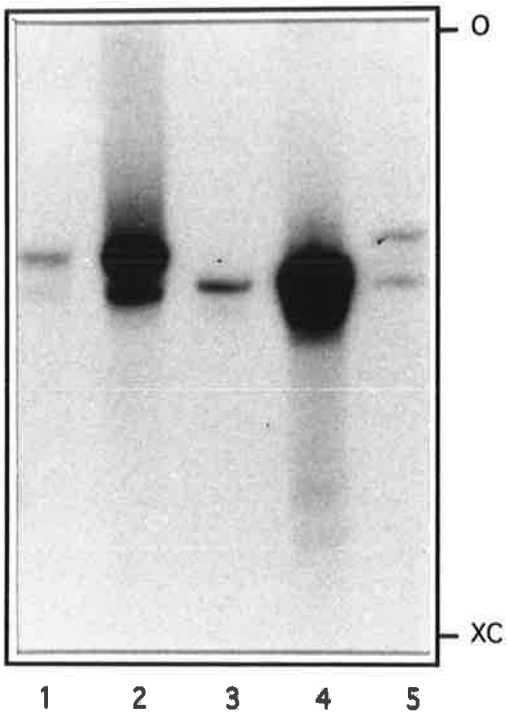
4.3.2. Confirmation of viroid profiles obtained by dot blot hybridization assay

Although the good correlation observed earlier between RT-PCR and dot blot hybridization results (See 3.3.3.2) confirmed the presence of viroids in the test samples, the correlation between the intensity of the dots and the actual concentration of the viroids remained to be established. Hence, Northern hybridization analysis was carried out on the total nucleic acids extracts of the Ramsey stock vine, its SAMC-derived vine R5 and the control vines Sultana H5, Shiraz BVRC30/BV8 and BVRC30/BV8/15 using ^{32}P-labelled GYSV1 riboprobes.

The results of Northern hybridization analysis (Fig. 4.2) showed the presence of two GYSV1 bands in each sample, with a slow-moving band of higher intensity corresponding to the circular viroids and a fast-moving band of lesser intensity corresponding to the linear viroids. The intensity of the fast-moving band was increased on incubation with 5 mM $MgCl_2$ at 80°C for 10 min, a treatment known to convert circular RNA to the linear form (Sänger et al., 1979).

The SAMC-derived vine R5 (Fig. 4.2, lane 1) showed a reduced GYSV1 band compared to its stock vine (lane 2), as observed by dot blot hybridization assay (Table 4.2). In contrast, the GYSV1 band from the micropropagated vine, BVRC30/BV8/15 (lane 4), was considerably stronger than that of its stock vine (lane 3), consistent with the dot blot hybridization results (Chapter 5, Table 5.1). The Sultana H5 stock vine (lane 5), had a GYSV1 level comparable to that of the BVRC30/BV8 stock vine (Chapter 5, Table 5.1). Variation in GYSV1 sequence was supported by the observation of GYSV1 bands at different positions in the three grape varieties, consistent with the reported existence of a large number of GYSV1 sequence variants in vines (Rigden and Rezaian, 1993, Polivka et al., 1996). The same GYSV1 variant bands were observed in the SAMC-derived Ramsey R5 and its stock vine (Fig. 4.2, lanes 1 and 2), different from those present in BVRC30/BV8, and its micropropagated vine BVRC30/BV8/15 (lanes 3 and 4), and Sultana H5 (lane 5).

Fig. 4.2. Northern hybridization analysis of GYSV1 content in SAMC-derived and micropropagated vines. The vine samples were in **lane 1**, Ramsey R5 regenerated by SAMC; **lane 2**, the Ramsey rootstock; **lane 3,** Shiraz BVRC30/BV8 stock vine; **lane 4,** BVRC30/BV8/15 micropropagated for 15 cycles; **lane 5,** Sultana H5 stock vine. Sixteen μg total nucleic acids (non-DNase-treated) were loaded in each lane and separated by 5% denaturing PAGE (7 M urea). Autoradiography was carried out by exposure to Fuji RX film for 72 h. In each sample, the more intense upper band and the less intense lower band corresponded to the circular and the linear forms of GYSV1 respectively. O indicates the position of the loading wells and XC, the position of the tracking dye, xylene cyanol.


O
XC
1
2
3
4
5

Northern hybridization analysis, carried out on the same samples with ^{32}P-labelled HSV riboprobes, showed a similar correlation of the intensity of HSV bands (data not shown) with the intensity of the hybridization dots obtained by dot blot hybridization. This confirmed that the viroid profiles obtained by dot blot hybridization (See 4.3.1.1) did in fact reflect the relative concentrations of the different viroids in each sample and were thus comparable to the viroid profiles previously reported by other workers (See 1.7.4).

4.3.3. Viroids in SAMC-derived vines grown in field conditions

To study further the behaviour of viroids in the SAMC-derived vines after transfer to field conditions, leaf samples were obtained from VAMVIA, from 50 SAMC-derived vines which had grown under normal vineyard conditions since 1991. These vines, when assayed by dot blot hybridization, gave a result essentially similar to that found for the PISA vines (data not shown). AGV was found in all 50 vines, GYSV1 in 48 vines and HSV in only three vines (Table 4.4).

An increase in the GYSV1 content was observed in two regenerants, compared to their respective stock vines, providing further evidence that increases in viroid titre could occur as a consequence of SAMC. Hence, the yellow speckle (YS) symptoms observed by Habili et al. (1992) on an FSAC-derived Arbane vine whose parental vine had previously been indexed as YS-free, could perhaps be a consequence of the FSAC process rather than of contamination in the field as suggested by the authors. A slight increase in AGV content was observed in five vines. None of the vines were observed to have an increased HSV content.

Two SAMC-derived vines, CS 125/R2A2S13 and CS 125/R2A8S2, were found to be negative for GYSV1. When both vines were re-assayed by RT-PCR using the optimised protocol for fast cycle times, both vines were found to be positive. Two rootstocks, Dog Ridge A6V8 and Paulsen 1103, were found negative for AGV by dot blot hybridization. However, this result could not be confirmed by the RT-PCR assay because the RNA extracts were used up on the hybridization analyses. These four vines

were the only vines found to be negative for GYSV1 and AGV, by dot blot hybridization assay, throughout this study.

4.3.4. A viroid profile typical for vines regenerated by meristem culture by dot blot hybridization assay

From the combined observations of SAMC and FSAC-derived vines from PISA and VAMVIA (Table 4.4), a pattern of GYSV1, HSV and AGV content emerged, from which a viroid profile, typical for vines regenerated by meristem culture, could be drawn. Such a profile would consist of a low level of GYSV1 and AGV detectable by dot blot hybridization with ^{32}P-labelled riboprobes and an HSV content reduced to a lower level that was detectable in most regenerated vines only by RT-PCR. Moreover, this profile was remarkably well maintained under *in vitro* and field conditions. This was confirmed by the unchanged viroid profile of CS LC30 L5, whether grown in the shadehouse or micropropagated (Chapter 5, Table 5.1). This viroid profile, typical for vines regenerated by meristem culture, was not observed in any of the 10 grape varieties (Chapter 3, Table 3.1), or the 32 stock and micropropagated vines (Chapter 5, Table 5.1) and the 24 stock vines used in this present study.

4.4. Discussion

4.4.1. Elimination of viroids in vines by meristem culture

The work described in this chapter provides the first comprehensive study carried out on vines regenerated by SAMC and FSAC in relation to viroid elimination by these two techniques. The results obtained with two diagnostic methods, dot blot hybridization and RT-PCR, both significantly more sensitive than the sPAGE used previously to index the putative viroid-free vines (Duran-Vila et al., 1988, Juarez et al., 1990), showed that viroids cannot be successfully eliminated in grapevines by meristem culture, whole or fragmented.

A summary of the data obtained by dot blot hybridization analysis on the 83 vines regenerated by SAMC and FSAC from 24 stock vines of 20 different varieties, including

Table 4.4: Dot blot hybridization assay of viroids in vines regenerated by SAMC and FSAC from PISA and VAMVIA

Source	Culture technique	Regenerant vines tested	GYSV1 +	GYSV1 -	HSV +	HSV -	AGV +	AGV -	CEV +	CEV -
PISA	**SAMC**	14	14	0	3	11	14	0	0	14
VAMVIA	**SAMC**	50	48	2	3	47	50	0	0	50
Total	**SAMC**	**64**	**62**	**2**	**6**	**58**	**64**	**0**	**0**	**64**
PISA	**FSAC**	19	19	0	6	13	19	0	0	19
TOTAL	**SAMC FSAC**	**83**	**81**	**2**	**12**	**71**	**83**	**0**	**0**	**83**

+, -, indicates the presence or absence of a hybridization dot respectively, using ^{32}P-labelled viroid specific riboprobes.

VAMVIA vines were grown in vineyards since 1991 whereas PISA vines were micropropagated or grown in the shadehouse.

Increased viroid titres were found in the following vines: GYSV in four SAMC-derived vines, two each from PISA and VAMVIA; HSV in one FSAC-derived vine from PISA, and AGV in one FSAC-derived vine from PISA and five SAMC-derived vines from VAMVIA.

rootstocks and wine grape varieties is given in Table 4.4. FSAC did not eliminate viroids as expected (Habili et al., 1992). The cumulative evidence obtained from the SAMC-derived vines from three different sources, WaggaWagga (See 3.3.2), PISA and VAMVIA, produced by two different SAMC techniques (Duran-Vila et al., 1988; Burrows and Ashton, 1993), in three different growth conditions, *viz. in vitro*, in the shadehouse and in the field, provided overwhelming evidence for the persistence of viroids in SAMC-derived vines. No cases of viroid-free vines were observed. The data indicate a differential reduction of viroid level rather than elimination as a consequence of meristem culture. A graduated response with a range of viroid contents, probably related to the size of the meristems excised, was found in the regenerated vines, in contrast to the total elimination of GYSV1 and HSV in every single regenerant vine (Table 4.1) reported by Duran-Vila et al. (1988). The vines found to have the lowest viroid content were the two GYSV1-negative vines in which AGV only was detected (data not shown). Three rootstocks, Teleki, Ruggeri 99 and Kober 5BB, and their regenerant vines maintained high GYSV1 titres (data not shown) as did the Ramsey vines from PISA (Table 4.2), indicating that it was particularly difficult to reduce the GYSV1 levels in stock vines with very high titres. However, HSV was no longer detected in any of these vines derived from rootstocks. Altogether, HSV was not detected in 71 vines of the 83 vines indexed (Table 4.4). Thus, the specific loss of HSV, as observed by the dot blot hybridisation assay, appeared to be a strong indicator for vines regenerated by SAMC and FSAC.

Thus, the production of disease-free plants by meristem culture (Morel and Martin, 1952), which is based on the original observation by Limasset and Cornuet (1949) that in systemically infected plants virus concentrations decrease as they approach the apical meristem, may not be directly applicable to viroid-infected plants. This could possibly be related to the location of viroids in nuclei and chloroplasts (Bonfiglioli et al., 1994, 1996).

The presence of viroids in the vines tested here, which, like the vines produced by Duran-Vila et al.(1988), were derived from parental vines grown under normal glasshouse conditions, is perhaps not surprising because meristem culture alone was

reported to be inadequate for viroid elimination (See Table 4.1) by Lizzáraga et al. (1980) and Paduch-Cichal and Kryczyñski (1987). A preliminary cold treatment of the stock vines for 4 to 6 months was reported to be essential for viroid elimination by both groups of workers. No details were provided by Juarez et al. (1990) when they reported that cold treatment had no effect on the production of viroid-free vines. Hence, the duration of the treatment could have had a critical influence on the result obtained. Future work aiming at the elimination of viroids in grapevines may require suitable treatment of the stock vines before attempting meristem culture.

4.4.2. Possible origin of the viroid profiles in vines regenerated by meristem culture

An attempt was made to provide an explanation for the viroid profiles observed in meristem-regenerated vines. The low level of viroids in the regenerated vines with the typical profile (See 4.3.4) could be due to the existence of a selection process in the apical meristem, such as the preferential association of certain low titre sequence variants with unknown components of meristematic cells, so that excision of the meristem tip would allow the transmission of only these sequence variants to the regenerants. Such a mechanism would be applicable to all three viroids studied, with HSV transmitted at a much lower level than GYSV1 and AGV. Evidence supporting the differential transmission of viroid variants was found in potato plants regenerated by axillary bud culture, following heat treatment, in which a severe strain of PSTV was eliminated but not a mild PSTV strain (Stace-Smith and Mellor, 1970).

From this perspective, only vines derived from the apical meristem tip would have this viroid profile. Larger apices would enable the transmission of viroid sequence variants not normally associated with the meristem tip; when micropropagated, these variants would multiply to different titres resulting in a range of viroid contents. The few regenerants detected with HSV could be a consequence of meristem slices slightly bigger that the meristem tips. Hence, although the greater number of HSV-positive regenerants

observed in FSAC-derived vines as compared to SAMC-derived vines may not be significant (Table 4.4), it could also reflect the larger sizes of apices used in FSAC.

The excision of excessively large apices, in which the total viroid complement is preserved, creates a situation whereby apices regenerate in conditions closer to micropropagation than to meristem culture and would account for regenerants, such as R8 (Table 4.2), with GYSV1 and HSV titres higher than in the stock vines. The presence of regenerants with increased GYSV1 titre, in the absence of HSV, such as L6, may possibly suggest a complex relationship between viroids where certain sequence variants with high titre could be favoured by the elimination of other viroids.

4.4.3. Decreased levels of AGV in the presence of high levels of GYSV1

A comparison of the viroid profiles of two FSAC-derived Ramsey vines may provide some evidence towards a previously unknown relationship between GYSV1 and AGV. A comparison of FSAC-derived vine R9, which had been micropropagated for 4 years, to its clone R7, which had grown in the shadehouse, showed an increase in GYSV1 titre in R9 from class 2 to 4 at the same time as a slight decrease in AGV titre from class 1 to 0.5 (Table 4.2). This decreased level of AGV, observed at the same time as an increased level of GYSV1 was also observed in L6 (Table 4.2). This effect, observed in the absence of HSV in both R9 and L6, suggested an influence of GYSV1 on AGV similar to that previously reported for HSV on AGV (Rezaian et al., 1988).

Both HSV and AGV replicated to high titres when gel-purified AGV preparations with trace amounts of HSV were inoculated into a cucumber host; however, HSV only was replicated using an inoculum with equivalent amounts of HSV and AGV, indicating the inhibition of the replication of AGV by HSV (Rezaian et al., 1988). A similar effect of HSV on CEV was observed in tomato (Rezaian et al., 1988). This effect of GYSV1 on AGV has not been reported previously.

The reverse effect, an increased level of AGV in the presence of a reduced level of GYSV1, was found in three field-grown vines from VAMVIA (Table 4.4), in which HSV was not detected by dot blot hybridization. Thus, the higher AGV titre observed in

the presence of high levels of both GYSV1 and HSV in Ramsey R8 (See 4.3.1.2), may indicate the presence of an interesting variant population of AGV that deserves further study.

4.4.4. Spread of viroids in vineyards

The maintenance of the viroid profile typical for SAMC-derived vines in most of the VAMVIA vines 4 years after transfer to normal field conditions suggested that the spread of viroids did not occur by normal vine maintenance procedures, as already suggested by Staub et al. (1995b) from the analysis of GYSV1 and HSV variants in neighbouring vines (See 1.7.3). However, the absence of HSV in the VAMVIA vines, as indexed by dot blot hybridization, would need to be confirmed by RT-PCR. It is feasible that there could be a low level of HSV in these vines that requires more time for the build-up of viroids to a level detectable by hybridization probes. This may be tested by the re-indexing of these vines by dot blot hybridization at a later stage.

Chapter Five

Viroids in Micropropagated Grapevines

5.1. Introduction

Grapevine micropropagation has been used as a means for the rapid clonal multiplication of new vine introductions, such as elite cultivars and virus-free stocks, to complement the traditional techniques of grapevine propagation through woody grafting and the rooting of cuttings. Micropropagation through tissue culture is the process whereby small pieces of plant tissue, e.g., apical meristems, leaf pieces and nodes, are multiplied on culture media and regenerated into complete plants (Bhojwani and Razdan, 1983).

In vitro propagation techniques for *Vitis vinifera* L. are already well established. Two methods for the clonal multiplication of vines have been described: (1) by multiple shoot formation through enhanced axillary branching using apical or axillary bud explants (Jona and Webb, 1978; Harris and Stevenson, 1982; Chee and Pool, 1982, 1983) and (2) by adventitious shoot formation from leaf primordial fragments (Barlass and Skene, 1978). The first method has generally been considered preferable because of the genetic stability of the progeny vines (Krul and Mowbray, 1984), although plants regenerated by the second method have also been shown to contain the normal chromosome complement (Barlass and Skene, 1978).

The principal consequences of *in vitro* culture on grapevines observed up to now have been an induction of juvenile characteristics, particularly in leaf morphology, and a reduced grape yield in micropropagated vines in the first few years after *in vitro* culture (Grenan, 1983, 1994; Deloire et al., 1995).

The effect of micropropagation on the numerous pathogens in grapevines has been less well studied. Pathogens in grapevines, of which the grapevine viruses are by far the most important, have been associated mostly with deleterious effects causing significant reductions in yield (Walter, 1988). Grapevine viruses are generally known for their negative influence, with infected vines showing slower growth, impaired root formation (Walter, 1988) and a multiplication rate reduced to varying degrees according to the type of infecting virus (Greño, 1988). Virus elimination in grapevines, obtainable by meristem culture (Galzy, 1972) especially in combination with heat therapy (Bass and

Legin, 1981), has been reported to have a mainly beneficial increase in yield (Bass and Legin, 1981; Valat et al., 1981). Although the elimination of certain grapevine viruses, e.g., grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV) and raspberry ringspot virus (RRV), has been reported after prolonged subculturing (Staudt and Kassemeyer, 1990, 1994), most viruses are not eliminated by micropropagation under normal conditions (Barba et al., 1989; Staudt and Kassemeyer, 1994).

To date, the role of viroids in grapevines is not known (See 1.7.6). The pervasive presence of viroids in all vines tested until now (Semancik, 1993a) has not allowed an assessment of the true influence of viroids in vines. Putative viroid-free vines of Cabernet Sauvignon, produced by shoot tip culture (Duran-Vila et al; 1988; Juarez et al., 1990), have not shown any significant differences so far when compared with viroid re-infected vines and vines containing naturally occurring viroid populations (Wolpert et al., 1996). Recent evidence indicates that pathogens may possibly have a positive contribution to grape quality (See 1.7.5). Hence, concern has been raised about the effect of micropropagation on grapevine viroids, which has not been studied so far, because of the importance of this *in vitro* multiplication step in the mass-propagation of cultivars in limited supply. In contrast to grapevine viruses, viroids are not eliminated in vines by meristem culture (Chapter 4). Hence, it is unlikely that viroids are eliminated under normal micropropagation conditions. However, it is relevant to investigate the behaviour of viroids during micropropagation to ensure that this multiplication step does not change the characteristics of the tissue culture-derived progeny vines as compared to the stock vines.

The aim of the work presented in this chapter was to study the effect of micropropagation on viroids in grapevines.

5.2. Materials and methods

5.2.1. Sources of plant material

The stock vines were obtained as follows: five vines BV2, BV7, BV8, BV9 and BV10 obtained as rooted cuttings from a Shiraz BVRC30 clone, originally obtained from

Orlando Wyndham, Barossa Valley. Five vines, Fd9, Fd25, Fd26, Fd28 and Fd29 of a Shiraz 12 clone, two vines, Sau6 and Sau25, of a Sauvignon Blanc F4V6 clone, two vines, Sem21 and Sem24, of a Semillon 32 clone and one Chardonnay vine, CD26, were obtained from the field collection of the Department of Horticulture, Viticulture and Oenology, University of Adelaide, Waite Campus. Sultana H5 and the GYSV1-, HSV- and AGV-negative control vines used in dot blot hybridization assays were obtained as described previously (See 3.2.2).

Four control vines, two micropropagated vines and their respective stock vines, were obtained from PISA through the help of Dr. R. Hamilton to study the effect of long term micropropagation on viroid content. The two stock vines were SAMC-derived L5 (See Table 4.2) and Fantasy Seedless, both planted in the shadehouse at PISA since 1991. Fantasy Seedless was originally imported as cuttings from Foundation Plant Materials Service (FPMS), University of California. Micropropagated samples were obtained from clones derived from both vines, cultured *in vitro* at PISA since 1991.

5.2.2. Establishment of vine cuttings in potting mix

Cuttings (15-20 cm in length with three buds) were obtained from the stock plants and struck by the bottom heat method of Nicholas et al. (1992) using the hot-bed facility of Dr. Brian Coombe, Department of Horticulture, Viticulture and Oenology, University of Adelaide, Waite Campus. Cuttings were cleaned and surface-sterilised in 25% ($^{v}/_{v}$) White King bleach (1% available chlorine) by standard procedures (See 2.3.1). They were then dipped for 40 s in the plant growth regulator β-indolebutyric acid (2 g/L), dissolved in absolute ethanol and diluted 1:1 with H_20, before planting in freshly steam-sterilised sand in a hot bed set up in a cold room kept at 4°C. Bottom heat was provided by a heating coil under the sand layer which maintained a temperature of 25-27°C. Under these conditions, bud swelling and rooting formation took place after 3-4 weeks.

After 4 weeks, the rooted cuttings were removed from the cold room and transferred to normal greenhouse conditions. Rooted cuttings were soaked in a 0.1%

($^{W}/_{V}$) solution of Benlate® DF fungicide (Du Pont de Nemours & Co., Delaware, USA) prior to potting into potting mix (See 2.4.1) in 20 cm plastic pots.

5.2.3. Micropropagation

5.2.3.1. Establishment of cultures *in vitro*

Nodal segments were obtained from vigorous young shoots with at least eight nodes growing in field or greenhouse conditions. Leaves were removed from the shoots at the base of the petioles and shoot segments (5-8 cm) were washed and surface-sterilized in 10% ($^{V}/_{V}$) White King bleach (0.4% available chlorine) for 10 min (See 2.3.1). After rinsing in sterile distilled water, they were cut into single node segments (1-1.5 cm in length) and dipped in an antioxidant mixture (ascorbic acid 100 mg/L, citric acid 150 mg/L) to reduce browning.

Explants (five per container) were cultured upright in 250 ml culture tubs in 25 ml of culture medium (See 2.3.2) consisting of MS salts at half strength, 10 g/L sucrose, 6.5 g/L Bacto agar and supplemented with 5 µM BA.

5.2.3.2. Shoot multiplication *in vitro*

When the original explants, as established in 5.2.3.1, reached 3-3.5 cm in length, shoots were excised, cut into single node segments and proliferated in 250 ml culture tubs in 25 ml of culture medium (See 2.3.2) consisting of MS salts at full strength, 30 g/L sucrose, 7.2 g/L Bacto agar and 5 µM BA.

After the first two subcultures, shoot multiplication was carried out in the same medium with MS salts replaced by C_2D salts. The C_2D basal medium was essentially based on the MS medium with the following modifications: $CaCl_2$ was substituted by $Ca(NO_3)_2$, KI was omitted and $MnSO_4$ was reduced from 100 µM to 5 µM. The C_2D organic constituents had the following composition: 3 µM thiamine HCl, 55.5 µM myo-inositol, 8 µM nicotinic acid, 5 µM pyridoxine HCl. Thereafter, multiplication was carried out by a two step-procedure involving root formation first followed by shoot proliferation. Shoots (12-15 mm in length, with two to three nodes) were excised from

the *in vitro* plantlets, cut 2-4 mm below the basal node and placed horizontally in a Petri dish (100 x15 mm), with the cut end submerged in a rooting medium consisting of C_2D basal salts (Chée and Pool, 1985, 1987) at half strength supplemented with 0.15 µM naphthalene acetic acid (NAA). After the appearance of root initials (after 4-10 days), the excised shoots were transferred to 250 ml culture tubs containing 25 ml of proliferation medium comprising C_2D basal salts at full strength, 30 g/L sucrose, 7.2 g/L Bacto agar and 5 µM BA and cultured under illuminated conditions (See 2.3.3). Subculturing was carried out every 6-8 weeks during the earlier cycles and 8-10 weeks in later cycles.

At each subculture, only 15 excised shoots were rooted, from which 10 of the more vigorous were cultured on the proliferation medium so as to keep the number of cultures at a manageable level.

5.2.3.3. Production of leafy cultures for RNA extraction

After 12-15 subcultures, the plantlets were grown to produce leaves for RNA extraction. They were transferred to 500 ml culture tubs containing 50 ml of basal C_2D medium, at full strength supplemented with 30 g/L sucrose, 7.2 g/L Bacto agar, 5 µM BA and 0.1% ($^{W}/_{V}$) activated charcoal. Growth over a period of three to four months produced enough leaves for RNA extraction.

5.2.4. Viroid assays

RNA extraction was carried out as described in section 4.2.2. Viroid RNA was extracted from the progeny vines directly from leaves grown *in vitro*. Viroid assays were carried out only by dot blot hybridization analysis using ^{32}P-labelled GYSV1, HSV, AGV and CEV full-length riboprobes and using the same negative controls as described previously (See 4.2.4). Hybridization was carried out in a single assay with the 10 grape varieties (Chapter 3), SAMC/FSAC-derived vines (Chapter 4), 11 seedlings (Chapter 6) and the cold-treated stock vines (Chapter 7).

5.3. Results

5.3.1. Micropropagation

The successful micropropagation of grapevines has been reported using the MS medium (Harris and Stevenson, 1982) and the C_2D medium (Chée and Pool, 1982). Single node explants were established in culture and multiplied on MS medium supplemented with 5 µM BA to enhance axillary branching. Shiraz BVRC 30 vines were used to develop the micropropagation procedure before applying it to the other grape varieties, hence frequent references to Shiraz are made in the text. A slow multiplication rate was observed on the MS medium with most nodes developing into a single shoot of five to six nodes in 6 weeks, with a massive callus at the base of the explants. After the appearance of the callus, the shoots grew very slowly and developed little further. After the first two cycles, the basal medium was changed from MS to C_2D, reported to be superior to MS (Chée and Pool, 1985, 1987), because of (1) the substitution of the chloride ions, known to be inhibitory to vine growth, by nitrate ions in the macro salts and (2) the decrease in manganese concentration and the absence of potassium iodide in the C_2D micronutrient formulation to reduce the inhibition of axillary proliferation. Growth of the *in vitro* vines was improved as a result of this change of medium. Leaves had a greener and healthier appearance although smaller in size, there was less basal callus on shoots and a higher rate of proliferation, consistent with that reported by Chée and Pool (1983). C_2D medium was therefore used for the remaining culture cycles for all varieties studied.

A two step-procedure was used for the multiplication process at each subculture because a faster proliferation rate was obtained when rooting was first induced in the excised shoots (Pool and Powell, 1975). After the appearance of the root initials, the explants were transferred to the shoot multiplication medium. Usually two to three short thick roots without secondary branching "rat tail roots" were formed. From the rooted explants, a rapid proliferation was observed as most axillary buds expanded rapidly to give clusters of new shoots.

Maximal shoot multiplication was observed for the Shiraz vines on C_2D medium supplemented with 5 μM BA. Shoots first developed from most axillary buds of the explant and then from the basal axillary buds of the new shoots. Each explant produced on average three to five shoots (2.5-3.5 cm in length) with five to six nodes per shoot in 6 weeks, with one to two small non-subculturable shoots which, on further incubation, elongated to the same size as the others. On average, a good multiplication rate was obtained with 20-30 nodes/excised shoot (with 4-7 mm internode length) in 5-6 weeks compared to 121 nodes/explant with cv. Remailly Seedless in 8-9 weeks on the same medium obtained by Chée and Pool (1987). Taking into consideration the varietal differences in response and the shorter subculturing time, the propagation rate was estimated to be quite reasonable.

The rate of shoot multiplication was slower in the other vine varieties. Semillon, Sauvignon Blanc and Chardonnay were most prolific when cultures were alternated on medium supplemented with 7.5 μM BA. Sultana was vitrified if grown continuously on medium supplemented with 5 μM BA media and had to be cultured alternately on medium supplemented with 2.5 μM BA.

The appearance of root initials in the Shiraz vines was increasingly delayed with the increasing number of subcultures. Roots were not formed in the other vines. Hence, the NAA concentration was increased from 0.15 to 0.4 μM to stimulate root formation in all varieties. In general, the growth rate and the proliferation rate slowed down and the subculturing intervals were increased from 6-8 weeks to 8-10 weeks.

The plantlets produced long internodes, large well-developed leaves and a normal root system after prolonged culture in the medium supplemented with 0.1% activated charcoal. Fully-regenerated vines were obtained from all the *in vitro* plantlets on transfer to the greenhouse (See 2.4.2). However, most of the vines were maintained *in vitro* and their leaves were used directly for RNA extraction.

5.3.2. Effect of micropropagation on viroid content

Assays were carried out only by dot blot hybridization assay and not by RT-PCR because the high levels of viroids were readily detectable by dot blot analysis and a better quantitative estimation of the levels of viroids was obtainable (See Fig. 4.2). A comparison of the GYSV1, HSV and AGV content in the stock and progeny vines, is shown in Fig. 5.1. The results, based on the rating of the intensity of the hybridization dots, are summarized in Table 5.1.

5.3.2.1. Viroid content in stock vines

An analysis of the viroid profiles in the 16 stock vines showed different viroid profiles in different grape varieties as expected but also in vines derived from the same clones, e.g., Shiraz BVRC 30 and Sauvignon Blanc F4V6 stock vines (Table 5.1). GYSV1 and HSV were found in all the stock vines except for HSV in the two control stock vines. Variations in both GYSV1 and HSV content were generally observed, with HSV at a higher titre than GYSV1. AGV was found at a low level in all the stock vines.

5.3.2.2. Viroid content in micropropagated vines

The viroid profiles of most micropropagated vines showed an increase in GYSV1 and HSV content (Table 5.1). An increase in GYSV1 content was observed in 14 out of the 16 progeny vines. The only two exceptions were Shiraz BV2 and Shiraz Fd26 which showed a decreased and an unchanged GYSV1 titre, respectively. . The increases in GYSV1 content differed between vines, even between those from the same clone. Thus, the Shiraz vines BV7, BV8 and BV9, which had the same initial level of GYSV1, showed a marked difference in GYSV1 titres after micropropagation (Table 5.1). GYSV1 in Shiraz BV8 increased from class 1 to class 4, while its clone Shiraz BV9 showed a minimal increase from class 1 to class 1.5. A similar effect was found in the Shiraz 12 and the Sauvignon Blanc micropropagated vines.

The changes in HSV content in the micropropagated vines as compared to the stock vines were essentially similar to those observed in GYSV1. All micropropagated

Fig. 5.1: Dot blot hybridization assay of stock vines and micropropagated vines

Stock and micropropagated samples used in the assays were as follows:

Row 1, Shiraz BVRC 30 stock vines BV2, BV7, BV8, BV9 and BV10; Shiraz 12 stock vines Fd 9, Fd 25, Fd 26, Fd 28 and Fd 29; Sultana H5 stock vine, Sult.

Row 2, Sauvignon Blanc F4V6 stock vines, Sau 6 and Sau 25; Chardonnay stock vine CD26; Semillon 32 stock vines, Sem 21 and Sem 24; and the two control vines, L5 and Fantasy Seedless table grapevine, Fan.

Rows 3 and **4,** the micropropagated vines (TC) arranged in the same order as their respective stock vines in rows 1 and 2.

1	BV2	BV7	BV8	BV9	BV10	Fd9	Fd25	Fd26	Fd28	Fd29	Sult
2	Sau6	Sau25	CD26	Sem21	Sem24	L5	Fan				
3	$BV2_a$	$BV7_a$	$BV8_a$	$BV9_b$	$BV10_b$	$Fd9_c$	$Fd25_b$	$Fd26_a$	$Fd28_a$	$Fd29_a$	$Sult_c$
4	$Sau6_c$	$Sau25_c$	$CD26_c$	$Sem21_c$	$Sem24_c$	$L5_d$	Fan_d				

The number of subculture cycles were in a, 15; b, 13; c, 12 and d, unknown. Both L5 and Fan, had been micropropagated for 4 years by PISA.

Only results for GYSV1, HSV and AGV are shown here since CEV was not detected in any sample. The negative control vines were as described in Chapter 4, Fig. 4.1.

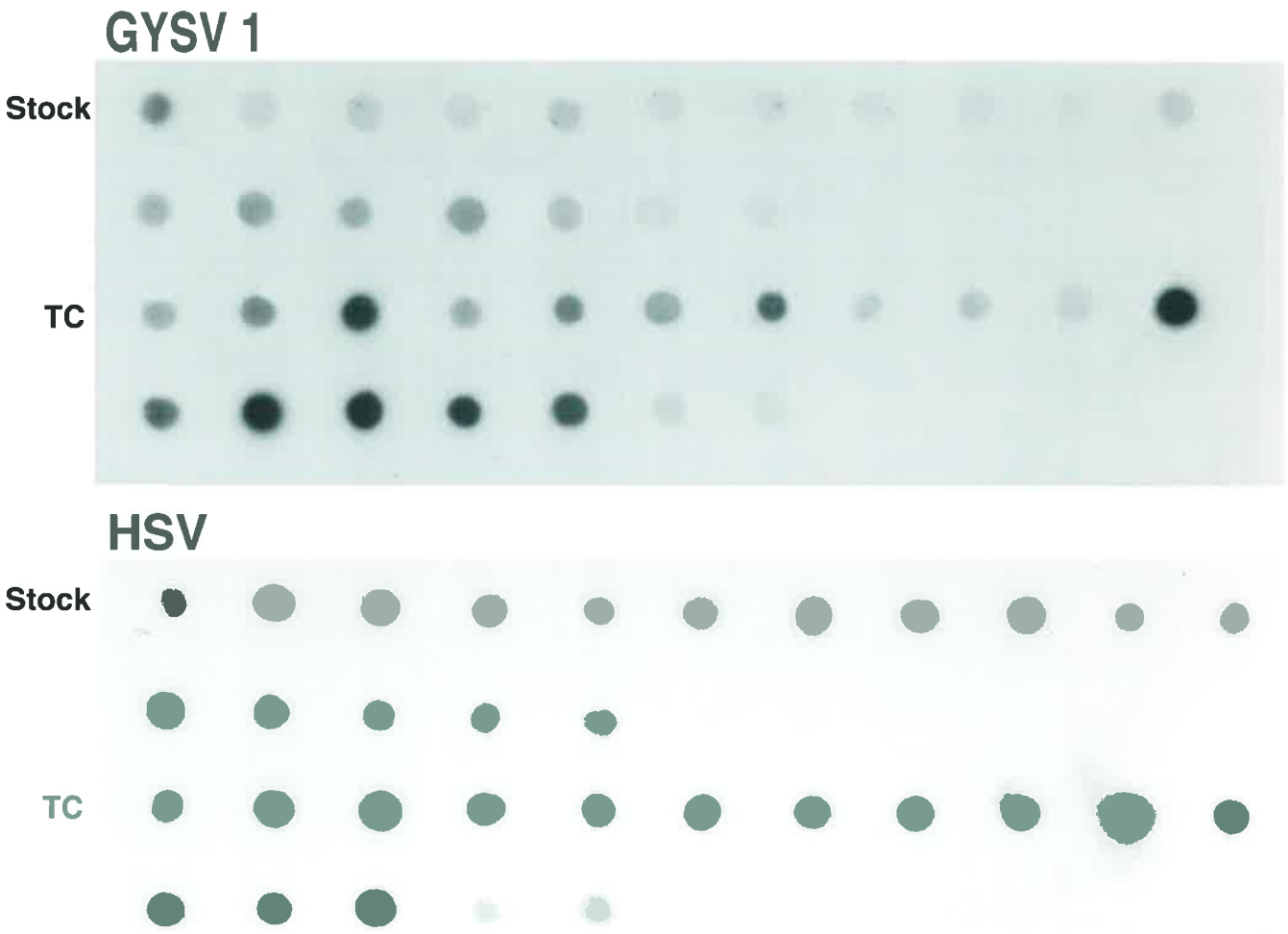

GYSV 1
Stock
TC
HSV
Stock
TC
AGV
Stock
TC

Table 5.1: Comparison of viroid profiles in stock vines and micropropagated vines by dot blot hybridization assay

Vines	No. s/c	GYSV1 stock	GYSV1 tc	HSV stock	HSV tc	AGV stock	AGV tc	CEV stock	CEV tc
Shiraz BVRC30/BV2	15	2	**1.5**	1.5	**3**	1.5	**0.5**	0	**0**
Shiraz BVRC30/BV7	15	1	**2**	3	**3.5**	1	**0.5**	0	**0**
Shiraz BVRC30/BV8	15	1	**4**	3	**3.5**	1	**0.5**	0	**0**
Shiraz BVRC30/BV9	13	1	**1.5**	2	**2.5**	1	**0.5**	0	**0**
Shiraz BVRC30/BV10	13	1.5	**2**	1.5	**2.5**	1	**0.5**	0	**0**
Shiraz 12/Fd9	12	1	**1.5**	1.5	**3**	1	**0.5**	0	**0**
Shiraz 12/Fd25	13	1	**2.5**	2.5	**2.5**	1	**0.5**	0	**0**
Shiraz 12/Fd26	15	1	**1**	2.5	**3**	1	**0.5**	0	**0**
Shiraz 12/Fd28	15	1	**1.5**	2.5	**3**	1	**1**	0	**0**
Shiraz 12/Fd29	15	1	**1.5**	2.5	**4**	1	**1**	0	**0**
Sultana H5	12	1	**4**	1.5	**3.5**	1.5	**1**	0	**0**
Sauvignon Blanc F4V6 Sau6	12	1.5	**3**	3.5	**3.5**	1	**1**	0	**0**
Sauvignon Blanc F4V6 Sau25	12	2	**4**	3	**3**	1	**1**	0	**0**
Chardonnay CD26	12	1.5	**4**	2	**3.5**	1	**1**	0	**0**
Semillon 32/Sem21	12	2	**3**	2	**1.5**	1	**1**	0	**0**
Semillon 32/Sem24	12	1.5	**3**	2	**2**	1	**1**	0	**0**
L5*	-a	1	**1**	0	**0**	1	**1**	0	**0**
Fantasy Seedless	-a	1	**1**	0	**0**	1	**0.5**	0	**0**

tc vines micropropagated through tissue culture.

s/c no. of subcultures over a 2-2.5 year period.

a no. of subcultures unknown; vines have been micropropagated since 1991.

* vine derived from SAMC.

Dot blot hybridization results were rated using the scale in Chapter 3, Table 3.2.

Although only one micopropagated vine was tested for each stock vine cultured, the viroid profiles of clones of the same grape varieties were found to be mostly comparable, both before and after micropropagation (Table 5.1), and different from those of the clones of other varieties. This suggested that the changes in viroid profiles observed after micropropagation were consistent and not likely to be an artifact.

vines showed increased HSV titres except for the two Sauvignon Blanc and the two Semillon vines. An identical high HSV content was found in the two Sauvignon Blanc vines, before and after micropropagation (Table 5.1), suggesting that there was probably a maximal titre attainable by a viroid population after which no further increases in viroid level could be obtained. The variable increases of GYSV1 and HSV content in the micropropagated vines most likely resulted from the differential replication of the viroid variants present in the stock vines (Visvader and Symons, 1986; Sano et al., 1992). The direct correlation between the increases in the intensity of hybridization dots and viroid concentrations in micropropagated vines, as confirmed by Northern hybridization analysis (Chapter 4, Fig. 4.2), ruled out the increases in dot intensity as artefacts due to the *in vitro* process.

In contrast to GYSV1 and HSV, AGV was reduced in eight out of the 16 micropropagated vines and maintained at the same level in the eight remaining vines. The reduced AGV content was consistent with the decreased levels of AGV observed in the presence of high levels of HSV (Rezaian et al., 1988) and GYSV1 (See 4.3.6). The reduction in AGV level could be variety-related since all eight vines with reduced AGV titres were Shiraz vines.

Insert ← The micropropagated control vines, L5 and Fantasy Seedless showed unchanged viroid profiles compared to their respective stock vines, except for a slight reduction of AGV in Fantasy Seedless. L5 maintained the viroid profile typical for SAMC-derived vines (See 4.3.4), confirming the absence of an influence of micropropagation on the viroid content in SAMC-derived vines. Fantasy Seedless, unexpectedly, also showed a similar profile. Subsequent verification of the origin of this vine with the FPMS, showed that this vine was not derived from meristem culture but had tested negative for all known viruses (of unspecified nature). Hence, the increase in viroid content, that could be expected after prolonged micropropagation from the observations reported above, was not observed. L5 and Fantasy Seedless were the only vines micropropagated over 4 years

that were available at the time of the experiment; the effect of SAMC on viroids in regenerated vines was not yet known.

5.4. Discussion

Sixteen vines of six different grape varieties were micropropagated to study the effect of micropropagation on viroid content in grapevines. Variable viroid profiles were found in the stock vines, even those derived from the same clone, consistent with the existence of different viroid variant populations in individual vines (Polivka et al., 1996). Micropropagation through tissue culture was found to result in a differential increase in the GYSV1 and HSV concentration in the majority of the micropropagated vines, most likely as a result of the increased viroid replication under the high temperature and high illumination conditions supplied during *in vitro* culture (Sänger and Ramm, 1974) rather than of the actual micropropagation process. Evidence in favour of this is provided by the higher incidence of detection of CCCV in coconut embryos germinated *in vitro* compared to seedlings germinated in nursery beds (Pacumbaba et al., 1993).

A reduction in rooting capacity and in growth rate, possibly related to the increase in viroid concentration, was observed with prolonged subculturing. This would be consistent with the reduced rooting capacity in the presence of increasing viroid concentration previously reported by Duran-Vila and Semancik (1982) and Takahashi et al. (1992). The converse effect, an increase in the rooting capacity, was observed after meristem culture (Duran-Vila and Semancik, 1986). These observations suggest that the change in viroid population under *in vitro* conditions may contribute to the decreased regenerative capacity normally observed after prolonged subculturing (Narayanaswamy, 1977).

Other dysfunctions could be induced in vines as a result of increased viroid levels but may not yet have been recognized as being viroid-related. One such possibility is the abnormal flowering in micropropagated vines as compared to vines produced by green grafting. A large scale evaluation of *in vitro* multiplied vines, carried out by Moët and Chandon, found losses of yield up to 50 % in Chardonnay and Pinot Noir as a

consequence of abnormal flowering after the *in vitro* process (Deloire et al., 1995). Abnormal flowering, believed to result from excessive vigour after *in vitro* growth, disappeared gradually after 7 years in the vineyard (Deloire, 1995). Grenan (1983) showed that abnormal flowering was related to grape variety. Of the ten grape varieties studied, Grenan (1983) found that seven varieties maintained a high yield in spite of the presence of juvenile leaf forms, suggesting that loss of yield was not necessarily associated with a return to juvenility. The remaining three varieties had a very low yield, with almost 100% reduction in one case.

The differential increases in viroid content observed after micropropagation in this study (Table 5.1) have certain similarities with the differential levels of abnormal flowering observed in the different grape varieties after micropropagation. This suggests that variations in viroid levels could possibly be involved in the abnormalities observed. Varieties with minimal changes in viroid levels would have a minimal reduction in yield after transfer to field conditions while varieties with viroids stimulated to high titres, as observed here in the Sauvignon Blanc, Chardonnay and Sultana vines (Table 5.1, class 4 intensity), would need a certain time lapse for the viroid titres to resume their normal levels under field conditions, before their normal production capacity could be restored.

Although this possible effect of increased viroid content on abnormal flowering in vines is at this stage purely speculative, it would be possible to test this easily in the case of the vines studied by Grenan (1983) by correlating the viroid profiles of the vine varieties before and after micropropagation with the effect on yield already observed. The re-indexing of the micropropagated vines obtained in this present study after a few years in the greenhouse may also provide information as to whether the altered viroid profiles are maintained as in the case of SAMC-derived vines or whether they resume their pre-micropropagation level. Limited evidence in favour of the second option was provided by a Viognier vine, which had been heat-treated at 35°C in the greenhouse for several months at CSIRO, Merbein, in the early 1970's to eliminate viruses and planted in a germplasm collection plot in the field at Merbein. Identical GYSV1 and HSV profiles were found in the stock Viognier and its heat-treated progeny by dot blot hybridization assay (data not

shown), more than twenty years after heat treatment. Although the initial viroid profile of the Viognier vine immediately after heat treatment is not known, an increase of viroids to high titres was likely from the observation of GYS symptoms on FSAC-derived Cabernet Franc vines regenerated at 35°C but not at 27°C (Barlass et al., 1982).

Hence, micropropagation conditions seem to act as an amplifying mechanism for differences in viroid populations that are not observable under normal circumstances. Micropropagation leads to a differential increase in the level of viroids in most vines by the selective amplification of certain viroid variants. This presumed effect of sequence variation could be confirmed by the cloning and sequencing of the viroid population extracted from specific vines before and after micropropagation to determine the change in viroid sequences within the viroid population. For a comprehensive study, a large scale sequencing exercise involving at least 20-30 clones needs to be carried out. The reduced or unchanged GYSV1 and HSV content observed in six micropropagated vines (Table 6.1) would need to be confirmed by replicate extractions and assays before any conclusion can be made.

This chapter needs much more expt. work to draw any satisfactory conclusions

Chapter Six

Viroids in Grapevine Seedlings

6.1. Introduction

Seed and pollen transmission of viroids are of considerable importance in the control of the spread of viroid-induced diseases (for review, see Mink, 1993). Of the 21 viroids found so far (Table 1.1), nine have now been detected either in the seeds or seedlings of one or more hosts. These are ASBV (Wallace and Drake, 1962), ASSV (Hadidi et al., 1991), CEV (Semancik, 1980), CSV and HSV (Kryczyñski et al., 1988), CCCV (Pacumbaba et al., 1993), CbV (Singh et al., 1991), and PSTV (Fernow et al., 1970). Five of these viroids, ASBV, CCCV, CSV, HSV and PSTV have also been detected in the pollen of infected plants (Desjardins et al., 1979; Pacumbaba et al., 1993; Kryczyñski et al., 1988).

Of the remaining viroids, no evidence of seed transmission was found for TPMV in tomato (Galindo et al., 1982), HSV in hops (Yaguchi et al., 1984), HLV in hops (Barbara et al., 1990), GYSV1 and HSV in grapevines (Taylor and Woodham, 1972; Semancik et al., 1987; Koltunow and Rezaian, 1988; Koltunow et al., 1988; Minafra et al., 1990). Hence, the transmissibility of viroids through seeds seemed to differ with the viroid species. Evidence obtained on PSTV, the best-studied of the seed-borne viroids, indicates that a host-specific factor may be involved causing a highly variable efficiency of PSTV transmission (5-100%) to progeny seedlings, depending on the host variety and the infecting viroid strain (Singh et al., 1992, 1993).

The first report on grape seeds as viroid-free germplasm was based on the observation that open-pollinated seedlings of Mission, normally used as a sensitive indicator variety for the biological indexing of grapevine yellow speckle (GYS) disease in the field because of its severe reaction to GYSV1, did not produce any diseased leaf symptom in the field (Taylor and Woodham, 1972). Subsequently, different workers using various indexing procedures failed to detect GYSV1 and HSV in grape seedlings derived from infected sources. Using sPAGE and silver staining for viroid indexing, Semancik et al. (1987) did not detect viroids in three Emperor seedlings derived from GYSV and HSV-infected stock vines. Nor did Minafra et al. (1990) detect GYSV1, GYSV2 and HSV in Mission and Aglianico seedlings, using the same technique. The

absence of GYSV1 in the Mission seedling was confirmed by dot blot hybridization analysis using ^{32}P-labelled cDNA probes prepared by reverse transcription of partly purified RNA extracts from infected vines. Northern hybridization analysis of RNA extracts of seedlings on a 2% agarose/formaldehyde gel using single stranded ^{32}P-labelled cDNA transcripts as hybridization probes also failed to detect GYSV1 in two Mission seedlings (Koltunow and Rezaian, 1988) and HSV in two composite samples prepared from 35 Mission seedlings and six Emperor seedlings, respectively (Koltunow et al., 1988).

Interestingly, the presence of viroids in seedlings was recorded in at least two instances: (1) GYS symptoms were observed on two plants of a seedling selection, cv. Carina, 4 years after its release by CSIRO, Merbein (Shanmuganathan and Fletcher, 1980), and (2) HSV was found in a Mission seedling, which originated from a seed stock known to produce only HSV-free seedlings when freshly germinated, 18 years after planting in the field (Koltunow et al., 1988). In both cases, the presence of viroids was interpreted as evidence for viroid spread in the field.

With the increasing sensitivity of updated detection techniques, more viroids are now being found in seeds and seed parts in which they were previously not detectable (Pacumbaba et al., 1993; Kryczyñski et al. 1988). Thus, the transmission of CCCV by pollen was demonstrated using PAGE and hybridization assays with ^{32}P-labelled riboprobes, providing the first evidence for a natural mode of spread of cadang-cadang (Pacumbaba et al., 1993). HSV and CSV, previously reported to be non-transmissible by seeds (Brierley, 1953; Dorst and Peters, 1974), were shown to be transmitted via both pollen and seeds in tomato by bioassay and 5% PAGE analysis followed by staining in 0.1% toluidine blue (Kryczyñski et al., 1988). Hence, it is not surprising that GYSV1, GYSV2, AGV and CEV were found in two Emperor table grape seedlings by RT-PCR assay, a result confirmed by dot blot hybridization assay in the case of GYSV1 and AGV (Chapter 3, see 3.3.3.2).

The aim of the work presented in this chapter was to confirm the transmission of viroids via grapevine seedlings.

6.2. Materials and methods

6.2.1. Sources of seedlings

Grape seeds were collected from identified vines from the field collection of the Department of Horticulture, Viticulture and Oenology, University of Adelaide, Waite Campus at the end of the May 1994 and kept in the fridge at 4°C sealed in plastic bags until use. Eight seedlings were obtained from seeds germinated aseptically on culture medium and maintained *in vitro* to exclude contamination by external sources. The seedlings and their respective parental vines were: PN1 and PN2 from Pinot Noir/V7; Sem1 and Sem2 from Semillon 32/Sem24; Merlot from Merlot/V11, Ries from Riesling/V26; Sau Bl from Sauvignon Blanc F4V6 Sau25; and Shz from Shiraz 12/Fd9. The Emperor seedling Vfr, was obtained as described previously (See 3.2.1). The Emperor seedling, RTG_2, was a clone of the *in vitro* germinated RTG (See 3.2.1), transferred to the greenhouse in 1994 and grown in isolation in a net box. The Mission seedling, Msn, selection AC 7448228 imported in 1974 from University of California, Fresno, by CSIRO, Merbein, was obtained from PISA through the assistance of Dr. R. Hamilton. It was maintained in the shade house together with a large number of other vines.

At the time of RNA extraction, Emperor RTG_2 and Emperor Vfr had grown in the greenhouse for 2 and 7 years respectively and Mission for 21 years in the shadehouse.

6.2.2. *In vitro* germination of grape seeds

6.2.2.1. Pre surface-sterilization procedures

Seeds stored at 4°C for several months before use were found to be more difficult to germinate than the fresh seeds of Emperor RTG germinated earlier. The seeds were soaked for 2-4 days in water through which a slow but constant stream of air was passed using an aquarium pump. Only seeds which sank in water and had increased in volume were used.

6.2.2.2. Surface-sterilization of grape seeds

Seeds were surface-sterilised by dipping in 90% ethanol for 20s and soaking in 25% ($^{V}/_{V}$) White King bleach (1% available chlorine) for 10 min on a shaker at 90 rpm. The seeds were drained by pouring through a sterile sieve and rinsed 3-5 times with SDW including at least 20 min on the shaker.

6.2.2.3. Culture of seedlings on sterile media

Sterilized seeds were cultured in 250 ml culture tubs containing 25 ml semi-solid agar medium consisting of C_2D salts at half strength and supplemented with 2% sucrose, 1 µM BA, solidified with 1.8 g/L Gelrite™ (Kelco) and 0.8 g/L Bacto agar (Difco). The seeds were maintained in the dark wrapped in foil for 10-30 days at 27°C and, on germination, were transferred to illuminated conditions (See 2.3.3). When seedlings were 3-3.5 cm high, they were transferred to 500 ml culture tubs in a medium containing 50 ml of basal C_2D media supplemented with 30 g/L sucrose, 5 µM BA, 0.1% ($^{W}/_{V}$) activated charcoal and 7.2 g/L Bacto agar. They were cultured for 7-9 months prior to viroid RNA extraction.

6.2.3. Viroid assays

Viroid assays were carried out (1) by dot blot hybridization assays using ^{32}P-labelled full-length riboprobes for GYSV1, HSV, AGV and CEV (See 4.2.4) in the same assay as the 10 grape varieties (Chapter 3), the SAMC/FSAC vines (Chapter 4), the micropropagated vines (Chapter 5) and the cold-treated stock vines (Chapter 7); (2) by Northern hybridization analysis after separation by 5% denaturing PAGE (7 M urea) (See 4.2.5) using ^{32}P-labelled full-length GYSV1 and HSV transcripts as probes, and (3) by RT-PCR using the optimized protocol for fast cycle times (See 3.2.5 and 3.2.6.2) and primer pairs c4h4 GYSV1 and c3h2 HSV with their optimal cycle parameters (Chapter 3, Table 3.1)

6.2.3.1. RT-PCR using the optimised protocol for fast cycle times and TaqStart™ Antibody

RT-PCR was carried out essentially as described in 3.2.5 and 3.2.6.2. TaqStart™ Antibody (Clontech) was diluted five fold in the dilution buffer provided (50 mM KCl, 10 mM Tris HCl, pH 7.0) and mixed thoroughly with *Taq* DNA polymerase, in the molar ratio recommended by the manufacturer, just prior to adding to the other reaction components in a bulk mixture. Aliquots of this PCR reaction mixture were added to the annealed primer pair/template mixture of each sample as previously and sealed individually in capillary tips as quickly as possible. Excessive delay (beyond 30 min) in starting the thermal cycling after the addition of the TaqStart™ Antibody to the *Taq* DNA polymerase was observed to have an inhibitory effect on amplification.

Amplification was carried out using c4h4 GYSV1 and c3h2 HSV primer pairs at their optimal T_a°C (Chapter 3, Table 3.1) and the number of PCR cycles increased to 40 for both GYSV1 and HSV.

6.2.4. Southern hybridization analysis

Southern hybridization analysis of the PCR products using ^{32}P-UTP-labelled cRNA probes (See 3.2.11) was carried out essentially as described in section 3.2.9. Membranes were washed after overnight hybridization at 65°C as follows: twice in 2xSSC, 0.1% SDS at RT for 5 min; twice in 0.2xSSC, 0.1% SDS at 65°C for 10 min and a final wash with 0.2xSSC, 0.1% SDS, preheated to 65°C, until the temperature reached RT. Autoradiography was carried out by overnight exposure to X-ray film (Fuji RX) at -80°C using an intensifying screen.

6.3. Results

Eleven seedlings from eight different grape varieties were analysed for their viroid content by three assay methods, dot blot hybridization assay, Northern hybridization analysis and RT-PCR. The viroid profiles of five of the parental vines, as assayed by dot blot hybridization, were already known from previous work: Pinot Noir/V7 and

Riesling/V26 (Chapter 3, Table 3.2), Semillon 32/Sem24, Sauvignon Blanc/Sau25 and Shiraz 12/Fd9 (Chapter 5, Table 5.1).

6.3.1. Dot blot hybridization assay of seedlings

The seedlings were indexed for GYSV1, HSV, AGV and CEV by dot blot hybridization assay using ^{32}P-UTP-labelled full-length transcripts as probes. The results (Table 6.1) show that GYSV1 was detected at a low, more or less comparable level in all 11 seedlings (Fig. 6.1a, Table 6.1). The lowest level was found in the Mission seedling.

HSV was detected in only two seedlings: *in vitro*-grown Shiraz at a very low level, and shadehouse-grown Mission at a very high level (Fig. 6.1b, Table 6.1). HSV was not detected in eight *in vitro*-grown seedlings and in greenhouse-grown Emperor Vfr. The absence of HSV in the micropropagated seedlings was consistent with previous reports of the absence of HSV in freshly-germinated grape seedlings (Koltunow et al., 1988). The high HSV infection found in the shadehouse-grown Mission seedling was also consistent with the previous report of HSV infection in a field-grown Mission seedling (Koltunow et al., 1988). Although the Mission seedling could have been infected by viroid spread in the shadehouse, this was unlikely from the work of Polivka et al. (1996) and because of the low GYSV1 content found at the same time in this Mission seedling. Selective contamination of the Mission seedling by HSV only without GYSV1 was ruled out by the fact that GYSV1 and HSV had been detected in almost all the vines indexed so far in this study. An alternative explanation, suggested by the presence of HSV in the Shiraz seedling, was the transmission of HSV at low level to the Mission seedling followed by the build-up of the HSV titre over the years. This possibility may be confirmed by re-indexing the Shiraz seedling at a later stage.

AGV was detected in all seedlings except Shiraz and Mission (Table 6.1) while CEV was not detected in any sample (data not shown).

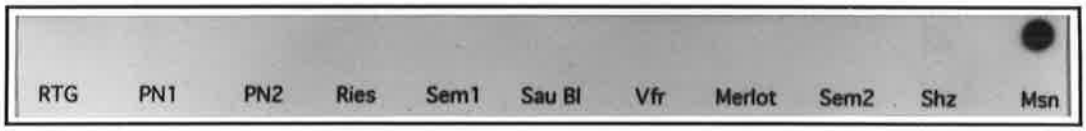
Fig. 6.1. Dot blot hybridization assay of GYSV1 and HSV in 11 seedlings. Assays were in (**a**) for GYSV1, (**b**) for HSV. Nucleic acid samples were from the following seedlings: RTG_2, PN1, PN2, Ries, Sem1, Sau Bl, Vfr, Merlot, Sem2, Shz and Msn. RTG_2 and VFr were grown in the greenhouse, Msn in the shadehouse; all other vines were germinated and maintained *in vitro*. Negative control vines were as described in Fig. 4.1.

Please note: The intensity of the hybridization dots of Shz and Msn decreased on photographic reproduction but were clear and obvious in the original autoradiograph.

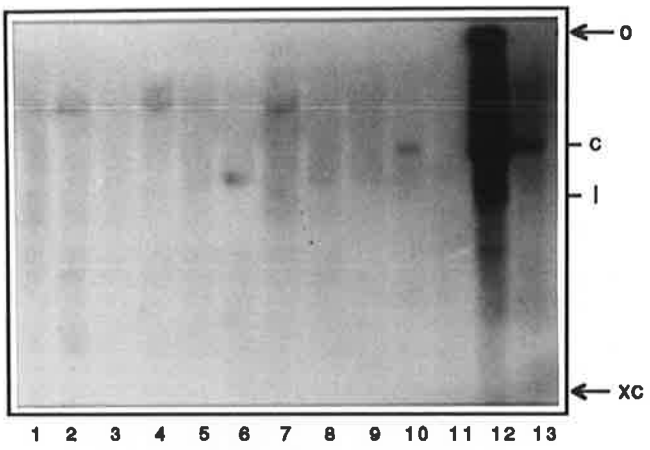
Fig. 6.2. Northern hybridization analysis of GYSV1 and HSV in 11 seedlings. Assays were in (**a**) for GYSV1, (**b**) for HSV. Nucleic acid samples were from the following seedlings: in **lanes 1-11**, RTG_2, PN1, PN2, Ries, Sem1, Sau Bl, Vfr, Merlot, Sem2, Shz and Msn. The control samples were, in **lanes 12 and 13**, the Riesling and Shiraz parental vines. Sixteen µg total nucleic acids (non-DNase-treated) were loaded in each lane and separated by 5% denaturing PAGE (7 M urea). Autoradiography was carried out by exposure to Fuji RX film for 120 h for GYSV1 and for nine days for HSV. o and xc indicate the position of the loading wells and the xylene cyanol tracking dye respectively. c and l refer to the circular and the linear forms of viroids in the control vines (**lanes 12 and 13**). The linear viroid band was shown by the increase in band intensity on incubation of RNA extract with 5 mM $MgCl_2$ at 80°C, a treatment known to convert viroids from circular to linear forms.

(a) GYSV1

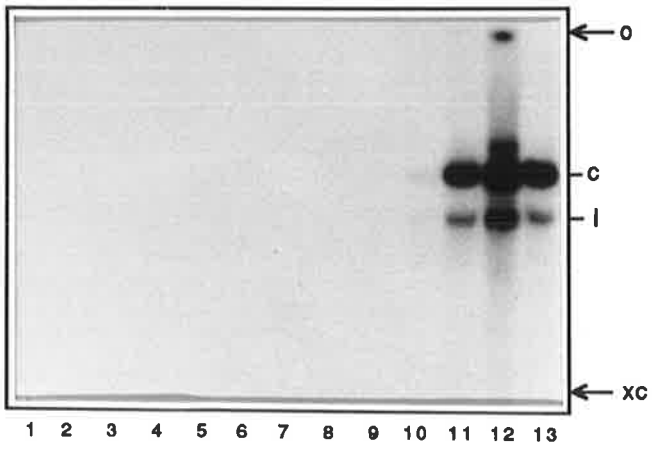
(b) HSV



(a) GYSV1



(b) HSV



6.3.2. Northern hybridization analysis of seedlings

Northern hybridization analysis of seedlings was carried out only for GYSV1 and HSV. The results (Fig. 6.2a and Table 6.1) show that GYSV1 was observed clearly in 10 seedlings, confirming the results already obtained by dot blot hybridization. However, the position of the viroid bands differed between the seedling varieties and the two parental vines. Viroid bands were observed at an identical position in seven seedlings, RTG_2, Vfr, PN1, PN2, Riesling, Sem1 and Sem2 (lanes 1-5, 7, 9), slower moving than the main viroid bands in the Riesling and Shiraz parental vines (lanes 12 and 13). The GYSV1 bands were found at a faster moving position only in the Sau B1 (lane 6) and the Shz seedlings (lane 10), in the latter case at a position corresponding to the circular viroid band of the parental Riesling and Shiraz vines (lanes 12 and 13). A GYSV1 band was not observed in the Mission seedling (lane 11), possibly because of the loss of RNA during Northern transfer.

HSV was detected only in the two seedlings, Shiraz and Mission (Fig. 6.2b, lanes 10 and 11, and Table 6.1), previously found to be HSV-infected by dot blot hybridization assay. The position of the HSV bands in both seedlings was identical to that of the HSV bands, circular and linear, in the stock Riesling and Shiraz vines (lanes 12 and 13).

6.3.2.1. Transmission of specific GYSV1 variants to seedlings

The detection by Northern analysis of a GYSV1 band in the Riesling seedling (Fig. 6.2a, lane 4) at a position different from that of the Riesling stock GYSV1 bands (lane 12), coupled with the presence of GYSV1 bands at an identical position in six other seedlings RTG_2, Vfr, PN1, PN2, Sem1 and Sem2 (Fig. 6.2a, lanes 1-3, 5, 7 and 9) suggests the transmission of specific GYSV1 variants to seedlings. Such an observation would not be unusual. The transmission of specific viroid variants via seeds had previously been suggested in other plant varieties (Fernow et al., 1970; Kryczyñski et al., 1988; Wallace and Drake, 1962). The presence of mild (Fernow et al., 1970) and symptomless strains of PSTV (Grasmick and Slack, 1986) was reported in potato

seedlings grown from PSTV-infected seeds. Studies on the transmission of PSTV, HSV and CSV through tomato seeds have shown the presence of all three viroids, by bioassay and 5% PAGE, in symptomless seedlings grown from diseased plants (Kryczyñski et al., 1988). Avocado seedlings, grown from symptomless infected trees, remained symptomless carriers of ASBV, while seedlings grown from symptomatic trees (Wallace and Drake, 1962) expressed strong symptoms.

The detection of specific GYSV1 variants (Fig. 6.2a), but not of HSV variants (Fig. 6.2b), in seedlings as suggested by the position of viroid bands on 5% denaturing PAGE (7 M urea), is consistent with the existence of a large number of GYSV1 variants compared to the few HSV variants reported previously (Rigden and Rezaian,1993; Polivka et al., 1996).

Additional support for the transmission of specific GYSV1 variants could be obtained by the comparison by Northern hybridization analysis of four of the six seedlings, PN1, PN2, Sem1 and Sem2 seedlings with their parental vines.

6.3.3. RT-PCR assay of seedlings

6.3.3.1. RT-PCR by the optimised protocol for fast cycle times

RT-PCR assay of the seedlings was carried out only for GYSV1 and HSV. A low efficiency of GYSV1 amplification was observed in all 11 seedlings using the c4h4 GYSV1 primer pair (data not shown). The GYSV1 bands in seedlings, as observed by Northern hybridization analysis, suggested that sequence variation as well as a low copy number of templates was influencing the amplification of GYSV1. Two approaches were unsuccessfully used to increase the yield of GYSV1 from seedlings. When the number of PCR cycles was increased from 25 to 30, a slight increase in yield was obtained, accompanied by the presence of numerous non-specific bands. The use of nested primer PCR (Haff, 1994) with the c1 GYSV1 primer for reverse transcription and the c4h4 GYSV1 primer pair for PCR (See Table 3.1) did not improve amplification.

HSV was detected only in Shiraz and Mission seedlings using the c3h2 primer pair. In the nine other seedlings, very faint PCR bands were observed which did not convincingly show the presence of HSV (data not shown).

6.3.3.2. RT-PCR by the optimised protocol for fast cycle times and TaqStart™ Antibody

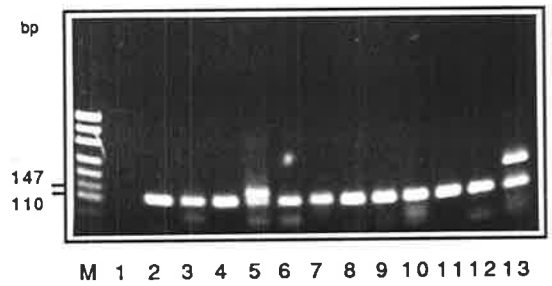
The RT-PCR assay of GYSV1 and HSV in the seedlings was successfully achieved only in the presence of TaqStart™ Antibody using an increased number of PCR cycles. An enhanced specificity had been observed earlier with TaqStart™ Antibody (See 3.3.2.6), a monoclonal antibody that effectively achieves a "Hot Start PCR" (Chou et al., 1992) by blocking *Taq* DNA polymerase activity at ambient temperature and dissociating from the enzyme at the start of thermal cycling to allow amplification to proceed optimally. Amplification of GYSV1 was carried out with the c4h4 GYSV1 primer pair, TaqStart™ Antibody and the number of PCR cycles increased from 25 to 40. The results (Fig. 6.3a and Table 6.1) show a high specificity and yield in the amplification of GYSV1 from all 11 seedlings, resulting in a very strong band of GYSV1-specific PCR product, confirmed by Southern hybridization analysis using ^{32}P-labelled riboprobes (Fig. 6.3c). The increase in band intensity was estimated to be at least 1000-fold higher than that obtained without TaqStart™ Antibody. In contrast, the intensity of the PCR product band for the high GYSV1 titre control vine, Sultana H5 (lane 13), was similar to that obtained without TaqStart™. However, a high molecular weight band, which had been observed as a faint band in earlier assays for GYSV1 by RT-PCR on Sultana H5 (Chapter 3, Figs. 3.5, 3.6 and 3.8), was amplified to high intensity (lane 13) in the presence of TaqStart™. A second high molecular weight band was also observed in the Riesling seedling (lane 5). Both bands were shown to be GYSV1 specific (Fig. 6.3c).

The RT-PCR assay was also carried out for HSV as above using the c3h2 HSV primer pair, TaqStart™ Antibody and 40 PCR cycles. The results (Fig. 6.3b and Table 6.1) show that HSV was present in all seedlings tested except Semillon Sem1 (lane 6). The yield of PCR product was higher in Shiraz and Mission (lanes 11 and 12) than in the

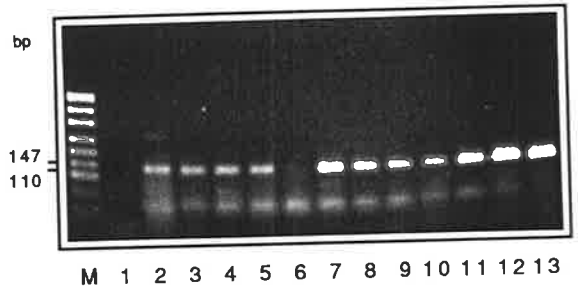
Fig. 6.3. RT-PCR and Southern hybridization assay of GYSV1 and HSV in eleven seedlings. RT-PCR assays were in (**a**) for GYSV1 and (**b**) for HSV. Amplification was carried out for 40 cycles using TaqStart™ Antibody and c4h4 GYSV1 and c3h2 HSV primer pairs and the T_a°C given in Table 3.1. Nucleic acid samples were from the following seedlings: in **lanes 2-12**, RTG_2, PN1, PN2, Ries, Sem1, Sau Bl, Vfr, Merlot, Sem2, Shz and Msn. In **lanes 1 and 13**, were the no template control and the positive control vine Sultana H5 respectively. The expected sizes of the PCR products were for GYSV1, 120 bp and for HSV 138 bp. PCR products (in 6 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments.

Southern hybridization analysis was carried out on the PCR products using ^{32}P-labelled full-length riboprobes in (**c**) for GYSV1 and in (**d**) for HSV. Autoradiography was carried out by exposure to Fuji RX film overnight for GYSV1 and 48 h for HSV.

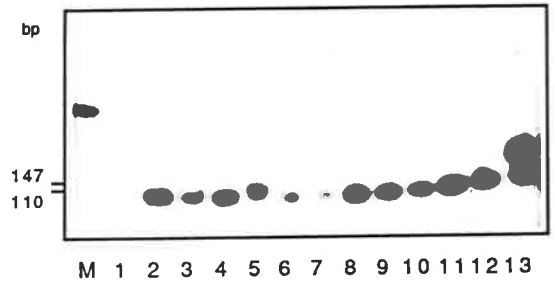
(a) GYSV1



(b) HSV



(c) GYSV1



(d) HSV

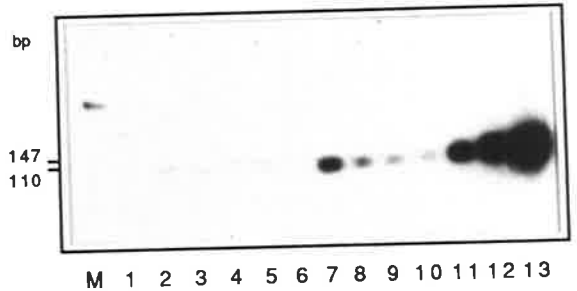


Table 6.1: The detection of viroids in seedlings by dot blot hybridization assay, Northern hybridization assay and RT-PCR.

Seedling	Source	GYSV1			HSV			AGV
		DB	NH	RT-PCR	DB	NH	RT-PCR	DB
Emperor RTG_2	greenhouse[a]	1	+	+	0	-	+	1
Pinot Noir PN1	*in vitro*	1	+	+	0	-	+	1
Pinot Noir PN2	*in vitro*	1	+	+	0	-	+	1
Riesling	*in vitro*	1	+	+	0	-	+	1
Semillon 1	*in vitro*	1	+	+	0	-	-	1
Sauvignon Blanc	*in vitro*	0.5	+	+	0	-	+	1
Emperor Vfr	greenhouse[b]	0.5	+	+	0	-	+	1
Merlot	*in vitro*	0.5	+	+	0	-	+	1
Semillon 2	*in vitro*	0.5	+	+	0	-	+	1
Shiraz	*in vitro*	0.5	+	+	0.5	+	+	0
Mission	shadehouse[c]	0.5	nc	+	2.5	+	+	0

+, -, indicates the presence or absence of a viroid specific band respectively.

DB, dot blot hybridization assay, was carried out for GYSV1, HSV, AGV and CEV; results were rated using the scale in Chapter 3, Table 3.2. CEV was not detected in any seedling.

NH, Northern hybridization assay, was carried out only for GYSV1 and HSV.

RT-PCR was carried out only for GYSV1 and HSV.

a-c, seedlings were grown for the following period: in a, 2 years; b, 7 years; and c, 21 years. All other vines were germinated *in vitro* and assayed within 9 months.

nc, not clear.

other seedlings. The identity of the PCR products was confirmed by Southern hybridization analysis using ^{32}P-labelled HSV riboprobes (Fig. 6.3d). The presence of HSV in Emperor Vfr was also shown by *in situ* hybridization techniques in conjunction with confocal laser scanning microscopy (D. Webb, personal communication).

6.3.4. A viroid profile typical for grape seedlings by dot blot hybridization assay

The present data suggest a common viroid profile for most of the seedlings tested, except for Sem 1, Shirax and Mission, similar to the viroid profile typical for SAMC-derived vines (See 4.3.2). Such a profile would consist of a low level of GYSV1 and AGV detectable by dot blot hybridization with ^{32}P-labelled riboprobes and a HSV content reduced to a level that was detectable in most seedlings by RT-PCR, as found in nine of the 11 seedlings tested, Shiraz and Mission excepted. The identical viroid profiles found in seven seedlings assayed nine months after *in vitro* germination and the two greenhouse-grown Emperor Vfr and RTG_2 vines, reinforced the similarity observed previously between SAMC-derived vines and seedlings. The viroid profiles of Shiraz and Mission seedlings suggest that seedlings with non-typical viroid profiles were also obtainable, as in SAMC-derived vines. The absence of HSV in Sem 1 could be due to a lower level of HSV in this particular seedling compared to other seedlings. Hence, Sem 1 needs to be rechecked at a later stage. (Was checked and found to be positive).

Further tests need to be carried out on a larger scale to confirm these observations obtained on a very limited number of seedlings.

6.4. Discussion

The presence of GYSV1, HSV and AGV was detected in 11 seedlings of eight different grape varieties by a combination of three different assay methods, dot blot hybridization assay, Northern hybridization analysis and RT-PCR, except for HSV in one case and AGV in two other seedlings. This is in contrast to previous work carried out mainly on seedlings of two grape varieties, Mission and Emperor, which were found to be viroid-free by other workers (See 1.7.6). The additional processing steps used in extracting viroid RNA from seedlings in the work described in all four references, that involved CF11 cellulose chromatography and salt

fractionation of nucleic acids (See 3.4), could have possibly contributed to the negative results.

The evidence obtained here suggests that the ubiquitous presence of viroids observed in all grapevines tested so far (Semancik et al., 1993) is extended to grape seedlings. The similar viroid profiles of grape seedings and SAMC-derived vines strongly suggest the transmission of viroids to seeds and to meristems via a common or identical mechanism. A high affinity of viroids with meristematic cells was shown by Kryczyñski (1983) during the transmission of viroids and viruses to inoculated plants by tissue implantation, with a rapid spread of viroids via plasmodesmata implied by the high efficiency (100%) of transmission of viroids from implants to host cells. Only viruses previously shown to be able to invade meristematic cells (Kryczyñski, 1983), were able to infect the inoculated plants via implants under the same circumstances. The absence of plasmodesmatal connections between the embryo and the adjacent cells (Bennett, 1969) has been suggested to account for the lack of transmission of most viruses to seeds (Zaitlin and Hull, 1987), and therefore, viroid transmission would be similarly inhibited. The increasing number of viroids detected in seeds (Mink, 1993) suggests that the localization of viroids in chloroplasts and nuclei (Harders et al., 1989; Bonfiglioli et al., 1994, 1996) may be responsible for the transmission of viroids to seeds and SAMC-derived vines.

The presence of a higher molecular weight PCR product, highly specific to GYSV1 as observed on several occasions in the Sultana H5 vine, raised the possibility of GYSV1 variants of higher molecular weight with end repeats as previously observed in the case of CCCV and CEV (Haseloff et al., 1982; Semancik et al., 1994). However the orientation of the c4h4 primer pair implied a repeat in the T1 terminal domain in contrast to the end repeats in the T2 terminal domains previously observed in CCCV and CEV. Preliminary studies carried out by Northern hybridization analysis on RNA extracts from three grapevine varieties, Sultana H5, Riesling and Pinot Noir, after separation on a 20% native PAGE as used by Hanold and Randles (1991a) to separate the different molecular

forms of CCCV, confirmed the presence of several high molecular weight bands similar to the higher molecular forms observed for CCCV (data not shown).

Since grapevines are propagated mostly vegetatively and because grapevine viroids have no obvious deleterious effect on their hosts, the seed transmission of grapevine viroids does not have the same practical importance as disease-causing viroids such as PSTV in potato and CCCV in coconut palms.

Chapter Seven

An Attempt to Produce Viroid-free Grapevines

7.1. **Introduction**

The indexing of meristem-regenerated vines, using two of the most sensitive diagnostic methods available for viroids, dot blot hybridization assay with ^{32}P-labelled riboprobes and RT-PCR assay (See 1.8.4-1.8.5), has shown that SAMC results in the differential reduction of viroids rather than their elimination (See 4.3.1.3) in grapevines grown under greenhouse conditions.

The production of viroid-free plants by SAMC in other crop species (Table 4.1) has already suggested that meristem culture alone may not be adequate to eliminate viroids (Lizárraga et al., 1980; Paduch-Cichal and Kryczyñski, 1987). However, evidence concerning the need for a preliminary treatment of stock plants at low temperature and low light intensity prior to meristem culture is contradictory, with viroid elimination favoured by such a treatment in potato and chrysanthemum (Lizárraga et al., 1980; Paduch-Cichal and Kryczyñski, 1987) but not in hops and grapevines (Momma and Takahashi, 1983, Juarez et al., 1990).

Viroid elimination from meristems has also been reported to be favoured by aiming for the minimal size of excised meristem fragments (Salazar et al., 1985). The highest percentage eradication of PSTV was obtained from apical domes, followed by meristem tips, comprising the apical dome and the first one or two leaf primordia only; the lowest percentage was obtained from shoot tips, which contained an additional two to three leaflets (Lizárraga et al., 1982). The high efficiency of viroid eradication using only the apical dome was presumed to be related to the limited vascular organization in this meristematic region and the absence, in rapidly dividing meristem cells, of plasmodesmata (Zaitlin and Hull, 1987) which constitute the major route for viral invasion. The size of meristem fragments excised could also possibly assume a greater importance in viroid elimination as compared to virus elimination because of the smaller size of viroids which would enable a more efficient invasion of meristematic tissues (Kryczyñski, 1983).

However, in view of the ubiquitous presence of viroids in vines (Semancik et al., 1993) and grape seedlings (Chapter 6) and the localization in chloroplasts and nuclei of the viroids studied so far (Harders et al., 1989; Bonfiglioli et al., 1994, 1996), the question that arises is whether it is at all possible to obtain viroid-free vines.

The objective of the work presented in this chapter was to attempt the production of viroid-free vines under conditions optimal for viroid elimination.

7.2. Materials and methods

7.2.1. Selection of stock vines

The four stock vines selected for SAMC were: the two seedlings, Emperor RTG_2 (Chapter 6, see 6.2.1) and Emperor Vfr (Chapter 3, see 3.2.1), and two SAMC-derived vines, CS L5 and Ramsey rootstock R5 (Chapter 4, Table 4.2). All four stock vines, as indexed by dot blot hybridization assay, showed either the typical viroid profile for grape seedlings (See 6.3.5) or the typical viroid profile for SAMC-derived vines (See 4.3.4). All four vines were growing in the greenhouse at 25-30°C prior to treatment at low temperature and low light intensity for viroid reduction.

Field-grown Shiraz 12 Fd9 (See 5.2.1) was the control stock vine.

7.2.2. Preliminary treatment of stock vines

The RTG_2, Vfr and CS L5 stock vines were transferred to a growth cabinet at 15°C/10°C with 16 h daylength of 2160 $\mu E\ m^{-2}\ s^{-1}$ intensity (Sun Lux Ace, NHT 360-LX, 4 lamps). The vines were fertilised every 2-3 weeks with Aquasol and treated for powdery mildew every 6-8 weeks. The latter treatment was carried out by alternately spraying with 3 g/L wettable sulphur (Sulfine, CropCare, ICI, UK) and 0.4 ml/L of the systemic fungicide, Bayleton (Bayer, Germany). At 2 months after treatment was started, the temperature was reduced to 13°C/10°C; at 5.5 months, the light intensity was further reduced to 1050 $\mu E\ m^{-2}\ s^{-1}$ (NHT 360-LX, 1 lamp). Treatment of the Ramsey R5 stock vine was started at 3 months when suitable material became available.

7.2.3. Surface-sterilization procedures and excision of meristems

Shoot tips (1-2 cm in length) were harvested from elongating shoots (8-10 cm in length) derived from newly-burst buds of the cold-treated vines. They were washed and surface-sterilised in 5% ($^{V}/_{V}$) White King bleach (0.2% available chlorine) for 5 min (See 2.3.1), and thoroughly rinsed at least five times in sterile distilled water, including a 20 min washing step on a shaker at 60 rpm. Residual chlorine in the explants through inadequate washing had been found to reduce the survival of the meristems in Stage 1 (See below).

The sterile shoot tips were dissected under a stereoscopic dissecting microscope (Zeiss Stemi 2000-C) with a fibre optic cold light source (Schott KL 1500) and using injection needles (0.51x25 mm, 25 G.1") for the excision of meristems. A fresh needle was used for each meristem to avoid contamination. To avoid desiccation, shoot tips were dissected in a Petri dish (100x15 mm) on sterile filter paper (Whatman 3MM, 30x30 mm) moistened with filter-sterilised antioxidant mixture (ascorbic acid 100 mg/L, citric acid 150 mg/L).

7.2.4. Meristem culture

Three *in vitro* culture steps were recognized in the regeneration of vines from meristems: Stage 1, the establishment of meristems in culture media; Stage 2, the regeneration of shoots from meristems; and Stage 3, shoot proliferation and rooting to produce fully regenerated vines.

7.2.4.1. Stage 1- Establishment of meristems in aseptic media

Seven different culture media, based on three different basal media (Chée and Pool, 1985, 1987; Emershad and Ramming, 1994; Lloyd and McCown, 1981), were used for Stage 1 culture of meristems (Table 7.1). All solid SAM culture media were gelled with 1.8 g/L Gelrite™ (Kelco) and 0.8 g/L Bacto agar (Difco) to give a semi-solid agar and dispensed into small Petri dishes (55 mm diameter) after autoclaving (See 2.3.2). The pH of both liquid and solid media was adjusted to 5.8 before autoclaving.

Table 7.1: Composition of media used for SAMC

Medium	Macro salts (strength)	% Sucrose	BA	AC	PVP 10	Liquid/ solid
STAGE 1						
SAM 1	0.5 X C_2D[1]	1.2	10 µM	—	—	L & S
SAM 2	**0.5 X C_2D[1]**	**1.2**	**5 µM**	**—**	**0.1 %**	**S**
SAM 3	0.5 X C_2D[1]	1.0	10 µM	—	0.1 %	S
SAM 4	1.0 X C_2D[1]	3.0	5 µM	—	—	S
SAM 5	0.5 X C_2D[1]	1.2	1 µM	0.1 %	—	S
SAM 6	1 X EmRa[2]	1.5	1 µM	0.3 %	—	S
SAM 7	1 X WPM[3]	1.5	1 µM	0.3 %	—	S
STAGE 2						
WPM 1	1 X WPM[3]	1.5*	5-10 µM	0.3 %	—	S
STAGE 3						
WPM 2	1 X WPM[3]	3.0	5-10 µM	0.3 %	—	S

AC, activated charcoal; PVP 10, polyvinylpolypyrrolidone 10.

L, S, liquid, solid.

[1], Chée and Pool, 1985, 1987.

[2], Emershad and Ramming (1994).

[3], Lloyd and McCown (1981).

*, glucose was used as carbon source instead of sucrose

The micronutrients and organic constituents were used as per formulation at full strength. In WPM 1, C_2D micronutrients and organic constituents were used at full strength. In WPM 2, C_2D micronutrients and Roubelaikis-Angelakis and Zivanovitc organic constituents (1991) were used, both at full strength.

SAM 2, the best medium for stage 1 culture is printed in bold.

SAM 4 was used for the routine micropropagation of vines (Chapter 5, see 5.3.1).

SAM 5 and 6 were used by Emershad and Ramming (1994) for the culture of immature embryos of seedless grapes.

Growth in liquid media was carried out either on filter paper bridges (Whatman 3MM) dipped in 5 ml medium in 35 ml culture tubes or on moist filter paper (Whatman 3MM) in 2.5 ml medium in Petri dishes (55 mm diameter) kept at a slant (~30°).

Freshly-excised meristems were exposed to low light intensity (30-50 $\mu E\ m^{-2}\ s^{-1)}$ with a 16 h photoperiod. After 10-15 days, meristems which had grown to 1x2mm-3x3 mm and started to turn green were exposed to illuminated conditions (See 2.3.3).

7.2.4.2. Stage 2- Regeneration of shoots from meristems

After 3-5 weeks, meristems which had developed to a size greater than 5x5 mm were transferred to 35 ml culture tubes in 10 ml of freshly-prepared medium. Two media were used: SAM 2 medium used for the earlier batches of meristems and WPM 1 medium for the later batches. The composition of the C_2D medium is given in section 5.2.3.2. The composition of WPM 1 medium was as follows: McCown's Woody Plant Medium (WPM) macro salts (Lloyd and McCown, 1981), C_2D micronutrients and organics (Chée and Pool, 1985, 1987), 15 g/L glucose, 0.3% ($^{w}/_{v}$) activated charcoal, 7.2 g/L Bacto agar and 5-10 µM BA, at pH 5.8. The WPM and C_2D macro salts differed mainly in their nitrogen content. The changes in composition of WPM as compared to C_2D were as follows: NH_4NO_3 (400 mg/L), K_2SO_4 (990 mg/L) and $CaNO_3$ (386 mg/L) in WPM compared to NH_4NO_3 (1650 mg/L), KNO_3 (1900 mg/L) and $CaNO_3$ (492 mg/L) in C_2D. Cultures were grown under illuminated conditions (See 2.3.3). The cultures developed slowly to give a single expanded shoot or a small cluster of shoots.

Excised shoots (1-1.5 cm in length) were transferred to 250 ml culture tubs containing 25 ml of the WPM 1 medium for further elongation.

7.2.4.3. Stage 3- Shoot proliferation and rooting

Elongated single shoots (2.5-3 cm) were transferred to 500 ml culture tubs for shoot proliferation and root formation in 50 ml WPM 2 medium (See Table 7.1) made up as follows: the basal WPM macro salts and C_2D micronutrients, with the Roubelaikis-Angelakis and Zivanovitc (1991) organic constituents (5 mg/L each thiamine, nicotinic

acid, pyridoxine HCl and pantothenic acid, 100 mg/L biotin, 100 mg/L myoinositol), 30 g/L sucrose, 0.3% ($^{W}/_{V}$) activated charcoal and 5-10 µM BA. Cultures were incubated under illuminated conditions (See 2.3.3).

The SAMC-derived vines from Stage 3 plantlets were conditioned in the greenhouse, as described in section 2.4.2, to produce fully regenerated vines.

7.2.6. Viroid assays

Viroid assays were carried out on the stock vines at different periods after the start of the treatment by dot blot hybridization assays using ^{32}P-labelled full-length riboprobes for GYSV1, HSV, AGV and CEV (See 4.2.4) in the same assay as the 10 grape varieties (Chapter 3), the SAMC/FSAC vines (Chapter 4), the micropropagated vines (Chapter 5) and the vine seedlings (Chapter 6). A separate assay for GYSV1 only was carried out for 13 of the 22 regenerated vines.

RT-PCR assays were carried out on samples of the stock vines using the optimised protocol for fast cycle times (See 3.2.6.2). The SAMC-derived vines were assayed by the optimised protocol for fast cycle times and TaqStart™ Antibody (See 6.2.3.1). Amplification was carried out using c4h4 GYSV1 and c3h2 HSV primer pairs at their optimal T_a°C (Chapter 3, Table 3.1) and the number of PCR cycles increased to 40 for both GYSV1 and HSV. Precautions against contamination were as descrbed previously (See 3.2.6.5). Two negative control samples without template were used in the assay for each viroid.

PCR products were analysed by Southern hybridization analysis (See 6.2.4) using ^{32}P-labelled riboprobes (See 3.2.11).

7.3. Results

7.3.1. Preliminary treatment of stock vines

Stock vines were treated at low temperature and low light conditions to reduce their viroid content (Sänger and Ramm, 1974). Cold treatment induced signs of senescence in all four vines, with mature leaves turning red or yellow and falling off the

vines. Of the four vines, Emperor Vfr was the most affected by the cold treatment and shed leaves at an early stage. At 3-4 months after the start of the cold treatment, bud burst took place followed by the vigorous growth of new shoots.

On reduction of light intensity, the stock vines became more sensitive to infection by powdery mildew and required more frequent treatment. The shoots grew longer internodes giving an etiolated appearance. However, all four stock vines survived 13 months of treatment reasonably well and were successfully returned to normal growth conditions in the greenhouse.

A continuous supply of new growth tissue, three to four apical buds at a time, was available for the duration of the experiment.

7.3.2. Regeneration of vines from meristems

The successful regeneration of vine apical meristems had previously been reported, both in liquid (Barlass and Skene, 1980a) and agar-solidified (Duran-Vila et al., 1988) MS medium supplemented with 10 μM BA. The regeneration of vines by SAMC from vines treated under the conditions described (See 7.2.2) was far more complex than that reported previously and three stages of *in vitro* culture were identified.

7.3.2.1 Stage 1- Establishment of meristems in culture media

7.3.2.1.1. Explant size

From a review of the literature, a disparity in terminology was apparent with the following terms used indiscriminately: shoot apical meristem culture, meristem culture, meristem tip culture, shoot tip culture and culture of shoot apices. The meristem, as defined by Quak (1977), is a dome (0.1-0.25 mm in length) of actively dividing cells that lies distal to the youngest leaf primordia. Because the rate of regeneration from the apical dome was reported to be extremely low, the 'meristem tip', as defined by Salazar et al. (1985), comprising the apical dome with one or two leaf primordia, was used as explant.

The excision of meristems was carried out on the basis of structure rather than size. Exceptionally large apical meristem tips were obtained from the rapidly growing

young shoots (5-8 cm in length) following new budburst. The sizes of these apical buds as compared to those derived from greenhouse-grown vines are shown in Fig.7.1a. Meristem tips (Fig. 7.1c), excised from both apical and axillary buds (Fig. 7.1b), showed a range of sizes (0.16-0.46 mm long), with an average size of 0.35 mm obtained from 14 meristem tips.

7.3.2.1.2. Liquid media vs solid media

Growth of meristems was faster on filter paper wicks dipped in liquid SAM 1 medium (Table 7.1) in a Petri dish than on filter paper bridges. The meristems expanded rapidly within 5-10 days to give distorted curled leaf-like structures similar to those described by Barlass and Skene (1978). On transfer to agar-solidified SAM 1 medium after 2 weeks, these leaf-like structures expanded further (25-30 mm in length). Although basal swellings were observed in the leaf-like structures, no development into shoots was observed, even after the ablation of most of the leaf blade to encourage regeneration (Barlass and Skene, 1980b).

On solidified SAM 2 medium, a slow but balanced development of the excised meristems was observed. The meristems maintained their original structure, developing very slowly and turning green (3x3 mm) after 15-20 days culture. After 8-10 weeks, the slow appearance of shoots was observed without the need for further intervention. Subsequently, the SAMC of all four stock vines was carried out on solid medium.

7.3.2.1.3. Meristem culture after 3-5.5 months of treatment

Meristems were excised from the cold-treated stock vines from 3 months to 7.5 months after therapy started. Five different solidified culture media, SAM 1 - SAM 5, were used in succession in an attempt to increase the survival rate of the meristems and to stimulate a better growth response (Table 7.1). No direct comparison of the five media was made because of the limited number of meristems obtainable at any one time. Hence the observed responses of the meristems to the different media were also influenced by the increasing duration of the treatment of the stock vines.

Fig. 7.1. Regeneration of vines from meristems

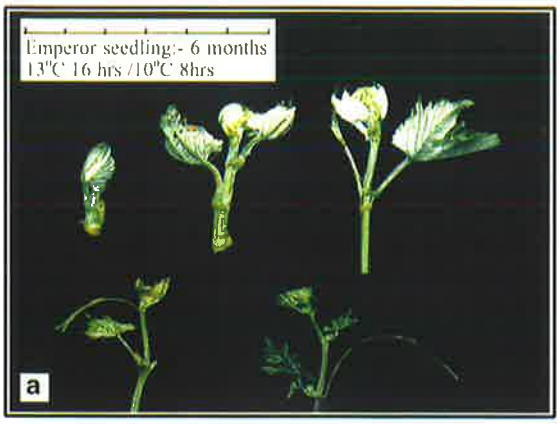
(a) Comparison of the sizes of apical buds of Emperor Vfr obtained from new budburst (top) after 6 months of treatment with the apical buds obtained from the same Vfr clone maintained in normal greenhouse conditions (bottom). Treatment of the stock vines was as follows using16 h daylength: 15/10°C, 2160 μE m^{-2} s^{-1}, for 2 months; 13/10°C, 2160 μE m^{-2} s^{-1} for 3.5 months, and 13/10°C, 1050 μE m^{-2} s^{-1} for 2 months. Divisions in scale bar: 1 cm.

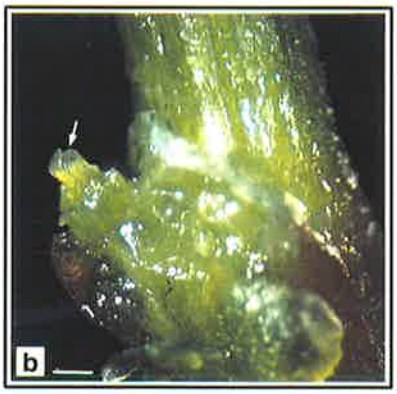
(b) An axillary bud (shown by arrow) with its outer layers removed. Scale bar: 1 mm.

(c) An exposed meristematic dome (d) and four leaf primordia (p). Scale bar: 0.2 mm.

(d) *In vitro* plantlets after Stage 3 culture, at transfer to the greenhouse for conditioning. From left to right, the vines were derived from Shiraz 12 Fd9, Cabernet Sauvignon L5, Emperor Vfr and Emperor RTG_2. Scale bar: 5 cm.

(e) The same four vines after growth in the greenhouse for 4 months. Scale bar: 7 cm.


Emperor seedling:- 6 months
13°C 16 hrs /10°C 8hrs
a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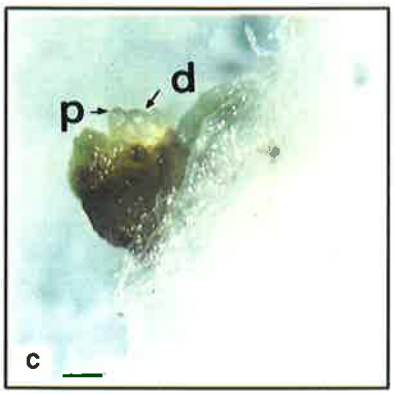

p
d
c


d


e

C_2D medium was selected for meristem culture because of its positive influence on vine micropropagation. C_2D macro salts were mostly used at half strength to accomodate the reduced nitrate metabolism reported in vines grown at low temperatures and to avoid toxic effects of nitrogen (Christensen et al., 1978). A very slow growth of the apical meristems was observed, in agreement with previous reports concerning the use of apical meristems as explants (Bhojwani and Razdan, 1983). The excised meristems of all four vines developed best on SAM 2 solid medium (Table 7.1), made up with C_2D salts at half strength, 12 g/L sucrose, 5 µM BA and 0.1% PVP 10 (Sigma) to reduce the oxidative effects of polyphenolic compounds. Growth was slower in SAM 1 solid medium than in SAM 2 medium. In SAM 3 medium, meristems showed a slight expansion before dying slowly. No growth was observed in SAM 4 medium, used routinely for the micropropagation of vines (Chapter 5, see 5.3.1), nor in SAM 5 medium. A high level of anthocyanins was observed in Emperor RTG in SAM 4 medium, with most of the meristems turning pink. This effect was not recognized at the time as an effect of the toxic level of nitrogen in the culture medium

7.3.2.1.4. Meristem culture after 5.5-7.5 months of treatment

With the increased duration of cold therapy, growth of the meristems slowed down markedly and the meristems displayed intense stress symptoms. These included anthocyanin formation, particularly pronounced in the two Emperor meristems, a lack of coloration in the CS L5 and a translucent appearance in the Ramsey R5 meristems. Prolonged culture on SAM 2 medium was required before transfer to Stage 2. The meristems which developed produced mostly small, stunted leaves, dried around the edges.

The poor condition of the meristems was further aggravated by the change to the low light condition at 5.5 months. Stress symptoms appeared within the first ten days of culture and the survival of meristems was drastically reduced. This problem was partly relieved by the maintenance of the cultures at low light intensity (30 µE m^{-2} s^{-1}) for a longer period (3-4 weeks) and transfer to the Stage 2 culture medium as early as possible.

No development was observed in cultures on SAM 5 and SAM 6 solid media (Table 7.1), which were based essentially on the media used by Emershad and Ramming (1994) for somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes. Meristems cultured on these media were rescued by transfer to SAM 2 and incubated at low light intensity until normal development was resumed.

7.3.2.2. Stage 2- Regeneration of shoots from meristems

Although the development of meristems into shoots took place in SAM 2 medium for meristems excised at early stages, meristems excised at later stages regenerated shoots only when cultured further in WPM 1, which had a nitrogen content 50% lower than SAM 2. This indicated the critical influence of the greatly decreased activity of nitrate reductase and decreased nitrate availability to the stock vines after long-term exposure to low temperature and low light intensity (Christensen et al., 1978) in the regeneration of plantlets from the meristems of treated stock vines. The symptoms of extreme nitrogen toxicity were progressively relieved with prolonged culture in WPM 1 medium.

Plantlets derived from Emperor Vfr developed only on medium supplemented with 10 μM BA, while plantlets derived from RTG_2 and CS L5 grew well on medium supplemented with either 5 or 10 μM BA and Ramsey R5 regenerated best on medium supplemented with 5 μM BA.

The initial culture conditions strongly influenced the subsequent development of the meristems. Hence, the meristems excised in the last batches, after the source of toxicity had been traced to the level of nitrogen in the culture media, and grown directly on WPM 1 media, regenerated first. The results (Table 7.2) show that less than a quarter (24.2%) of the meristems which were established in culture in Stage 1 grew successfully to stage 2 as a result of heavy losses incurred by culture on SAM 2 medium.

7.3.2.3. Stage 3- Shoot proliferation and rooting

Both shoots and roots developed rapidly in WPM 2 medium to give regenerated vines with multiple shoots and an extensive root system (Fig. 7.1d). Of the 63

meristems which developed to Stage 2, 23 (36.5%) proliferated to give multiple shoots and roots on WPM 2 medium to give vine plantlets. This was equivalent to 8.8% of the total number of meristems established in culture (Table 7.2). Of the 12 CS L5 and seven RTG_2 plantlets that survived through Stage 3, four CS L5 and two RTG_2 were derived from meristems excised after 7.5 months of treatment and grown directly in SAM 2 medium at Stage 1 followed by WPM 1 at Stage 2. These represented the regeneration, respectively, of 100% and 50% from the last batch of meristems excised. The four Vfr plantlets, which survived to Stage 3, were all derived from meristems excised after 4 months of treatment of the stock vines. None of the Ramsey vines developed beyond Stage 2, consistent with the previously reported difficulties of regenerating Ramsey vines from meristems (Burger and Schumann, 1990).

Of the 25 surviving plantlets at Stage 3, 22 fully regenerated vines were obtained after conditioning in the greenhouse (Fig. 7.1e). In summary, 7.1% of the total number of meristems excised or 8.5% of the meristems established in culture were fully regenerated (Table 7.2).

7.3.3. A protocol for the regeneration of vines from meristems treated at low temperature and low light intensity

From the above observations, the following protocol is recommended for the regeneration of plants from meristems derived from vines grown for a prolonged period under the low temperature and low light intensity conditions described: **Stage 1**: Excised meristem tips are grown in agar solidified SAM 2 medium at low light intensity (30 $\mu E\ m^{-2}\ s^{-1}$) for 2-4 weeks until the meristems start to turn green. They are then transferred to conditions of increased light intensity (200-350 $\mu E\ m^{-2}s^{-1}$) for 2 weeks until meristems reach 5x5 mm in size. **Stage 2:** Expanded meristems are transferred to WPM 1 medium for 6-8 weeks until elongated shoots are formed (1.5-2.5 cm in length). **Stage 3**: Excised single shoots are transferred to WPM 2 medium for 4-6 weeks for shoot proliferation and root development before conditioning in the greenhouse.

Table 7.2. Regeneration of vines by SAMC from treated stock vines

Stock vines	Meristems excised	Losses[a]	No. meristems cultured at Stage 1	Stage 2	Stage 3	No. vines regenerated[b]	% vines regenerated[c]
CS L5	156	20	136	19	14	12	8.8%
Emperor Vfr	56	12	44	22	4	4[d]	9.1%
Emperor RTG_2	58	8	50	14	7	6	12.0%
Ramsey R5	38	8	30	8	0	0	0%
Total	**308**	**48**	**260**	**63**	**25**	**22**	**8.5%**
% survival[e]	100%		84.4%	20.5%	8.1%	7.1%	
% regeneration[f]			100%	24.2%	9.6%	8.5%	

[a] Losses due to fungal infection derived from the stock vines.

[b] Vines fully regenerated in the greenhouse.

[c] Percentage vines regenerated from meristems successfully established in culture at Stage 1.

[d] All four Vfr vines were regenerated from meristems excised after 4 months of treatment.

[e] Percentage survival of meristems as a function of total number of meristems excised.

[f] Percentage survival of meristems as a function of total number of meristems established in culture.

7.3.4. Assay of viroids in stock vines

The viroid profiles of all four stock vines as indexed by dot blot hybridization assay were already known before treatment was started: Emperor RTG_2 and Vfr (See Chapter 6, Table 6.1); CS L5 and Ramsey R5 (See Chapter 4, Table 4.2) and Shiraz 12 Fd9 (See Chapter 5, Table 5.1). Further, the presence of HSV in Emperor RTG_2 and Vfr seedlings had been shown by RT-PCR (See 6.3.3.2).

Dot-blot hybridization assays of the four treated stock vines were carried out for GYSV1, HSV, AGV and CEV at different times (3, 4, 6.5 and 7.5 months) after the start of the treatment to monitor the viroid levels before meristem excision. GYSV1 and AGV were detected in all four vines at levels decreasing with the duration of treatment (data not shown) while HSV and CEV were not detected, as expected from the initial viroid profiles. The presence of GYSV1 in CS L5 at 4 months and in all four treated vines at 6.5 months was confirmed by RT-PCR assay using the optimised PCR protocol for fast cycle times (data not shown).

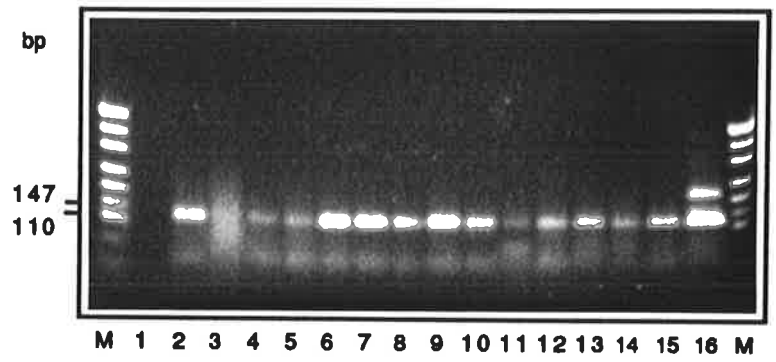
Dot-blot hybridization assay of the four stock vines, after treatment for 13 months, carried out on a composite sample of six actively growing shoot tips (1 cm in length) from each stock vine, showed that GYSV1 was reduced to the limit of detection in all four vines (data not shown). AGV was not assayed.

7.3.5. Assay of viroids in SAMC-derived vines

Dot blot hybridization assay was carried out for GYSV1 on 13 of the 22 vines regenerated by SAMC and which had grown in the greenhouse to produce enough leaves for extraction. The samples assayed were: three RTG_2-derived vines, RTG R1-R3; five CS L5-derived vines CS C1-C5; five Vfr-derived vines, Vfr V1-V5, and one Shz M4 vine, derived from the control stock vine, Shiraz (Table 7.3). The results showed the presence of GYSV1 in all regenerated vines. The level of GYSV1 in the regenerated vines was similar to that in the stock vines prior to treatment (See Table 4.2 and Table 6.1). Assay was not carried out for HSV because all four treated stock vines were known to be HSV-negative.

Fig. 7.2. RT-PCR assay of GYSV1 in vines regenerated from the meristems of treated stock vines. The samples were, in **lanes 2-15**, RTG R1-R3, CS C1-CS C5, Vfr V1-V5 and Shz M4 respectively. In **lanes 1 and 16** were the negative control without template and the positive control vine Sultana H5 respectively. Amplification was carried out using TaqStart™ Antibody, c4h4 GYSV1 primer pair, T_a 58°C and 40 PCR cycles. The expected size of the PCR product was 120 bp. PCR products (in 6 µl) were separated on a 2% agarose gel. The DNA size markers (**lanes M**) were pUC 19/*Hpa*II fragments.

Fig. 7.3. RT-PCR assay of HSV in vines regenerated from the meristems of treated stock vines. The samples were, in **lanes 2-15**, RTG R1-R3, CS C1-C5, Vfr V1-V5 and Shz M4 respectively. In **lanes 1 and 16** were the negative control without template and the positive control vine Sultana H5 respectively. Amplification was carried out using TaqStart™ Antibody, c3h2 HSV primer pair, T_a 56°C and 40 PCR cycles. The expected size of the PCR product was 138 bp. PCR products (in 6 µl) were separated on a 2% agarose gel. The DNA size markers (**lane M**)were pUC 19/*Hpa*II fragments.


bp
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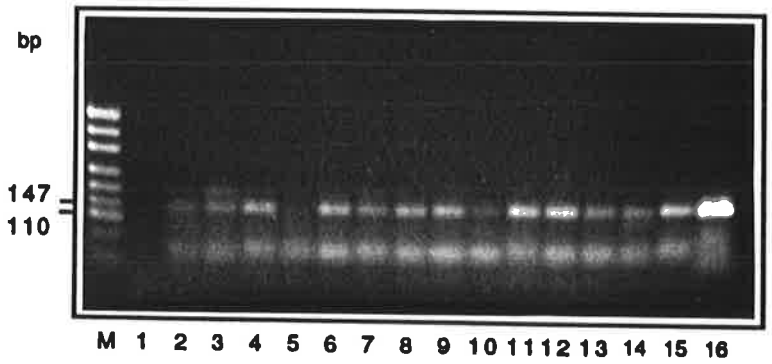
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M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M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Table 7.3: Dot blot hybridization and RT-PCR assay of vines regenerated from meristems of treated stock vines

Sample code[a]	SAMC-derived vine[b]	Duration of treatment[c] (months)	GYSV1		HSV	
			DB	RT-PCR	DB	RT-PCR
RTG R1	RTG_2 M11A2	4	1	+	nt	+
RTG R2	RTG_2 M22A3	7.5	1	+	nt	+
RTG R3	RTG_2 M22A2	7.5	0.5	+	nt	+
CS C1	CS L5 M5A1	3.5	1	+	nt	nc
CS C2	CS L5 M16A1	5	1	+	nt	+
CS C3	CS L5 M16A2	5	1	+	nt	+
CS C4	CS L5 M18A1	6	1	+	nt	+
CS C5	CS L5 M22A2	7.5	1	+	nt	+
Vfr V1	Vfr M13A3	4.5	0.5	+	nt	+
Vfr V2	Vfr M13A1	4.5	0.5	+	nt	+
Vfr V3	Vfr M13A1	4.5	0.5	+	nt	+
Vfr V4	Vfr M13A2	4.5	0.5	+	nt	+
Vfr V5	Vfr M13A4	4.5	0.5	+	nt	+
Shz M4	Shz M4	3	1.0	+	nt	+

a , simplified code given to b, the SAMC-derived vines as labelled in the greenhouse.

c, length of treatment of stock vines at the time meristems were excised. Treatment of the stock vines was as given in Fig. 7.1.

+, -, indicates the presence or absence of a viroid specific band respectively.

DB, dot blot hybridization assay, was carried out only for GYSV1 because the stock vines had already tested negative for HSV. Results were rated using the scale in Table 3.2.

nt, not tested; nc, not clear.

Vfr V2 and V3 were clones produced from the same SAMC-derived vine.

RT-PCR assay of the SAMC-derived vines, carried out using the optimised protocol for fast cycle times and TaqStart™ Antibody, detected the presence of GYSV1 (Fig. 7.2) and HSV (Fig. 7.3) in most of the vines tested, confirmed by Southern hybridization analysis (data not shown). The expected GYSV1 bands were found in all the SAMC-derived vines (Fig. 7.2, lanes 2, 4-14) except in RTG R2 (lane 3) where a long smear was obtained. However, GYSV1 was most likely to be present in this vine as shown by the dot blot hybridization results (Table 7.3). The most intense GYSV1 bands were observed in RTG R1 (Fig. 7.2, lane 2) and CS C2-C5 (lanes 6-9).

HSV was detected in all SAMC-derived vines (Fig. 7.3, lanes 2-4 and lanes 6-14) except in the CS C1 vine (lane 5). Since this vine was already found to be GYSV1-infected both by dot blot hybridization and RT-PCR assay (Table 7.3), this meant that none of the SAMC-derived vines tested were viroid-free.

7.4. Discussion

An attempt was made, using a coordination of factors known to reduce viroid titre, to produce viroid-free vines. Vines were regenerated by SAMC from four stock vines, two seedlings and two SAMC-derived vines of three different varieties, selected for their low viroid content and known viroid profiles and treated under low temperature and low light conditions known to favour the reduction of viroid content (Sänger and Ramm, 1974). A low percentage regeneration of vines (8.5%) was observed from meristems excised from cold-treated stock vines (Table 7.2), consistent with the low survival of explants derived from vines grown in conditions of stress (Harris and Stevenson, 1982) and the low rate of regeneration obtained from meristems of cold-treated plants of other species (Momma and Takahashi, 1983). However, the regeneration of vines from the treated meristems could be substantially increased by culture on the appropriate media.

Of the 22 vines regenerated, 13 were indexed for GYSV1 and HSV by dot blot hybridization assay and RT-PCR; all the vines tested were found to be viroid-infected. The evidence obtained here indicates that viroids cannot easily be eliminated from

grapevines by SAMC. Although one vine was found to be HSV-free, this was most likely due to an HSV level that was too low to be detected even with this RT-PCR protocol with increased sensitivity.

However, the persistence of viroids in grapevines was not unexpected considering the localization of viroids in nuclei and chloroplasts (Harders et al., 1989; Bonfiglioli et al., 1994, 1996). The efficiency of elimination of viral pathogens from grapevines was previously shown to differ according to the type of virus and to the growth conditions of the diseased vines resulting in a differential elimination of grapevine viruses (Bass and Legin, 1981; Habili et al., 1992; Staudt and Kassemeyer, 1994). This elimination was possibly related to the size, molecular structure and mode of transmission of the viruses involved (Bass and Legin, 1981). Hence, the conditions for the elimination of the phloem-restricted grapevine leafroll-associated closteroviruses (GLRaV) were reported to differ from those for the smaller grapevine fan leaf nepoviruses (GFLV) which were not limited to vascular tissues and invaded very young meristematic tissues (Goussard and Wiid, 1993; Koruza et al., 1993). While the elimination of grapevine closteroviruses was achievable by *in vitro* culture techniques alone (Altmeyer, 1990; Goussard and Wiid, 1993), the elimination of GFLV was reported to require a preliminary heat treatment (Gifford and Hewitt, 1961; Harris and Stevenson 1979; Monette, 1986). In the case of viroids, the lack of a protein coat as well as the absence of a tertiary structure and their intracellular localization, may possibly explain why viroids are not eradicated under conditions known to eliminate viruses.

The previous reports of viroid-free plants from other crop species could perhaps have been a consequence of the limited sensitivity of the viroid detection techniques used (See Chapter 4, Table 4.1) to index the putative viroid-free plants. The specific elimination of severe viroid strains (Stace-Smith and Mellor, 1970), leaving behind mild viroid strains that persist in low titre in a symptomless condition (Kryczyñski et al., 1988), could be a further contributive factor. Alternatively, SAMC-derived plants of other crop species could be genuinely viroid-free, and the presence of viroids in SAMC-derived grapevines could be linked to some essential function in grapevines so far

unknown. This issue could possibly be resolved by the use of more sensitive viroid detection techniques to re-index the putative viroid-free plants of other crop species.

Chapter Eight

Application of the high sensitivity RT-PCR Assay to the Diagnosis of Grapevine Leafroll-associated Viruses

8.1. Introduction

Grapevine leafroll (GLR) is one of the most important grapevine diseases worldwide causing a significant reduction in both crop quality and yield (Woodham et al., 1984; Goheen, 1988). GLR has been found to be associated with at least six serologically different strains of clostero-like viruses (Gugerli et al., 1984; Rosciglione and Gugerli, 1986; Hu et al., 1990a, b; Zimmermann et al., 1990a, b; Boscia et al., 1995). The grapevine leafroll-associated viruses 1 and 3 (GLRaV-1 and GLRaV-3) have been found to be by far the most widespread and the most consistently associated with GLR (Boscia et al., 1995). Until recently, GLR was believed to be spread only by vegetative propagation. However, GLRaV-3 was reported to spread naturally between vines (Rosciglione and Gugerli, 1989; Tanne et al., 1989; Habili et al., 1995), with mealybugs (Rosciglione and Gugerli, 1989; Tanne et al., 1989) and scale insects (Belli et al., 1994) as transmitting vectors. This has raised serious concern since there is currently no method to control the spread of the disease. Reliable and sensitive techniques for virus detection, independent of seasonal fluctuations of viral titre and the uneven distribution of viruses in infected tissues (Minafra et al., 1992; Monis et al., 1994), are essential for the control of GLR.

A review of the techniques generally used in virus diagnosis and their relative sensitivities has recently been made by Matthews (1993); those specifically applicable to the detection of grapevine viruses were reviewed by Monis (1994). So far, serological techniques have been the most widely used for the detection of leafroll viruses, with antibodies for GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-5 being commercially available.

RT-PCR techniques have already been applied to the detection of GLRaV-3 (Minafra et al., 1994; Levy et al., 1994). However, only a minor increase in sensitivity over hybridization techniques was observed because of inhibition of RT-PCR by grapevine RNA extracts (Minafra and Hadidi, 1994). Two approaches were used, with limited success, to increase the sensitivity of RT-PCR assays: (1) the immunocapture-RT-PCR (IC-RT-PCR) technique (Wetzel et al., 1992) which required the preliminary capture

of virions on microtitre plates before the release of viral RNA as template, and (2) the use of the Gene Releaser™ polymeric matrix (BioVentures, USA) to bind the inhibitors in the RNA extract (Levy et al., 1994). However, IC-RT-PCR did not yield the expected sensitivity (Minafra and Hadidi, 1994) and a low efficiency of amplification was obtained from RNA extracts prepared by Gene Releaser (Levy et al., 1994).

The objective of the work presented in this chapter was to apply the sensitive RT-PCR protocols developed for the diagnosis of grapevine viroids to the detection of GLRaV-3 and possibly to GLRaV-1.

8.2. Materials and methods

8.2.1. Sources of plant material

GLRaV-3-infected vines were obtained through the kind assistance of Dr R. Hamilton, PISA. The GLRaV-3 positive control vines used were: Tinta Carvahla and Pinot Noir (PN) Antav 543 rooted from cuttings obtained originally from the Nuriootpa (South Australia) variety collection. Samples from both vines previously tested positive for GLRaV-3 by ELISA (A. Ewart, personal communication). As part of a research program conducted by PISA, two backyard varieties were identified by ampelography*, as likely to be Frullo and Olasz, and were subjected to biological indexing to determine their health status. CF-Frl and CF-Olz, obtained by green-grafting (Cirami et al., 1988) Frullo and Olasz respectively to Cabernet Franc indicator vines, showed variable GLR symptoms under field indexing conditions and were obtained from PISA for this study. Such "variable response" vines usually develop GLR symptoms only in years with cool and moist seasons during the main ripening phase of grapes (R. Hamilton, personal communication). The putative negative control vines were CS LC10, a South Australian selection made in the early 1980s and previously found to be free from GLR, and Shiraz BVRC30 BV9 (See 5.2.1). All six vines were maintained in the greenhouse, University of Adelaide, Waite Campus.

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*ampelography, the science of grapevine identification by observation and measurement of grapevine characteristics.

The rootstocks tested were: Ramsey 1 and Ramsey 812, obtained from Loxton Centre, South Australia and Ramsey HR16 and Black Malaga TGR7 obtained from Nuriootpa. Leaf samples were obtained from the rootstocks growing in field conditions.

Leaf samples of GLRaV-1 infected vines were kindly provided by Dr. N. Habili, CSIRO Division of Horticulture, Adelaide. The positive control vines were: a Cabernet Franc CF-LR1 infected by graft-inoculation from a low yielding Sultana clone and Sultana B4L, a low yielding clone from Adelaide. Both samples previously tested positive for GLRaV-1 by Western blot analysis and ELISA (N. Habili, personal communication). The negative control vine was the seedling Emperor Vfr (See 3.2.1).

8.2.2. Viral RNA extraction

Grapevine cortical tissues (0.5-1.0 g) were stripped from grapevine petioles and ground in a cold mortar in 10 ml of the viroid extraction buffer (See 3.2.2). The fine slurry was transferred to a 10 ml tube and centrifuged at low speed (100 g) for 5 min in a bench centrifuge. The supernatant was transferred to a 15 ml Corex tube and 1 ml of TE_1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 10 µl 2-mercaptoethanol and 65 mg sodium sulphite was added, followed by 1 ml 10% ($^{w}/_{v}$) SDS. After incubation for 10 min at 60°C in a water bath, 3.2 ml of 5 M K acetate were added, and the tube incubated on ice for 30 min. The solution was centrifuged at 11,000 rpm for 10 min in a Sorvall HB_4 rotor at 4°C for 10 min. The supernatant was transferred to a fresh Corex tube and the nucleic acids precipitated by the addition of an equal volume of isopropanol and incubation for 1 h at 4°C. The pellet was resuspended in 2 ml TE_2 buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) and extracted with acid phenol as follows: 200 µl of 2 M sodium acetate, pH 4.0, 1.8 ml water-saturated phenol was added to the suspension which, after vortexing thoroughly, was incubated on ice at 4°C for 10 min. Two ml of $CHCl_3$ were added to this solution. After vortexing carefully, the suspension was spun at 11,000 rpm in the Sorvall HB4 rotor at 4°C for 10 min. The aqueous layer was transferred to a fresh Corex tube and re-extracted with $CHCl_3$. The RNA in the aqueous layer was precipitated by the addition of one volume of isopropanol and incubation

overnight at -20°C. After washing with 70% ethanol, the white pellet was briefly dried under vacuum before resuspension in TE_2 buffer. The RNA extract was purified by the passage of 25-50 µl aliquots through a Sepharose CL-6B spin column (See 2.2.4).

8.2.3. RT-PCR assay for GLRaV-1 and GLRaV-3

RT- PCR was carried out in capillary tips using the optimised protocol for fast cycle times as described in section 3.2.6.2. The primer pairs used for GLRaV-1 and GLRaV-3, the optimal parameters for amplification and the sizes of the expected products were as given in Table 8.1.

The GLRaV-3 primer pair was as published by Minafra and Hadidi (1994). The putative GLRaV-1 primer pair was designed by Dr. N. Habili, from the sequence of a cDNA clone pLR1, derived from a low-yielding Sultana B4L clone (Habili and Rezaian, 1995) and which hybridised specifically to the dsRNA from a number of grapevine varieties infected with GLRaV-1.

8.2.4. Confirmation of the identity of the 340 bp PCR product amplified with the GLRaV-3 primers

The amplified 340 bp PCR product was purified (See 2.2.3) and cloned into pGEM $5Zf^+$ vector (See 2.2.9) to give the clone Zgl 9. After sequence analysis of the clone Zgl 9 (See 2.2.10), the PCR primer sequences were excised by *Mse*1(See 2.2.1) and a 116 bp long internal fragment was used as template for the preparation of ^{32}P-labelled cDNA probes by the random priming method (See 3.2.8). Southern hybridization analysis of the GLRaV-3 cDNA clone, pGLR 9ds, was carried out after separation on a 2% agarose gel, using these cDNA probes. Clone pGLR 9ds, containing a 1100 bp fragment derived from GLRaV-3 and inserted into pUC 18 (Saldarelli et al.,1994), was originally obtained from Dr. G. P. Martelli and eventually provided by Dr. N. Habili.

Table 8.1: Primers for the RT-PCR assay of GLRaV-1 and GLRaV-3 and parameters for optimal amplification

Primer	Sequence	T_a°C	No. cycles	Product length (bp)
c GLRaV-3	5' ATT AAC TTG ACG GAT GGC ACG C 3'			
h GLRaV-3	5' ATA AGC ATT CGG GAT GGA CC 3'	59	27	340
c GLRaV-1	5' TTC CGT GTC TAA CAT CCG TC 3'			
h GLRaV-1	5' ACG TTG AGA TTA GTC TGA CTC 3'	58	30	400

T_a, annealing temperature.

8.2.5. Southern hybridization analysis of the PCR products

Southern hybridization analysis (See 3.2.9) was carried out on the PCR products, after separation on a 2% agarose gel and transfer to Hybond N^+ membrane, using ^{32}P-dCTP-labelled cDNA probes prepared by random priming (See 3.2.8) from the 1100 bp insert of the pGLR 9ds clone.

8.2.6. Dot blot hybridization assay

Dot blots were prepared from total RNA extracts as described in section 3.2.10 and hybridization assay (See 3.2.9) carried out using ^{32}P-dCTP-labelled cDNA probes prepared by random priming (See 3.2.8) from the 1100 bp insert of pGLR 9ds clone.

8.3. Results

8.3.1. Extraction of grapevine leafroll viral RNA

In preliminary experiments, the RT-PCR assay of GLRaV-3, using nucleic acid extracts of GLRaV-3 infected leaves prepared by the viroid RNA extraction protocol, had been observed to be unsuccessful. Hence, the viroid extraction protocol was modified for the extraction of viral RNA as follows:

(1) Grapevine cortex tissues were used instead of leaves because of the presence of GLRaV in high concentration in the phloem tissues (Martelli, 1993).

(2) The supernatant obtained after the first low centrifugation spin to remove the cell debris was not fractionated at high speed as carried out for viroid extraction. Instead, it was treated directly with SDS to release the viral RNA.

(3) DNA was removed from the crude nucleic acid extracts by an acid phenol extraction (Kedzierski and Porter, 1991), a step previously found to produce efficient templates for PCR analysis by McKittrick (1993).

(4) Viral RNA was further purified by passage through a Sepharose CL-6B spin column to remove low molecular weight molecules inhibitory to the RT-PCR assay. A low amplification efficiency was observed without this last step.

This extraction protocol, suitable for fresh and frozen tissues, gave an average yield of 220 µg RNA/g fresh weight tissue with a A_{260}/A_{230} ratio of 1.33. RT-PCR assay for GLRaV-3 was carried out successfully on RNA extracts obtained from 10 grapevine varieties. This extraction protocol also yielded large quantities of viroid RNA from viroid-infected vine tissues, as shown by dot blot hybridization analysis. However, viroid amplification was not efficient using RNA extracts from this modified protocol when compared to extracts prepared by the viroid RNA extraction protocol (See 3.2.2).

8.3.2. Development of an RT-PCR assay for GLRaV-3

The RT-PCR assay for GLRaV-3 was developed using PN Antav 543, found to be positive for GLRaV-3 by both ELISA and dot blot hybridization assay using ^{32}P-labelled cDNA probes. The optimal cycle parameters for the amplification of GLRaV-3 using the GLRaV-3 primer pair were derived as described previously (See 3.3.2). The best amplification was observed with a T_a of 59°C and 27 PCR cycles (Table 8.1).

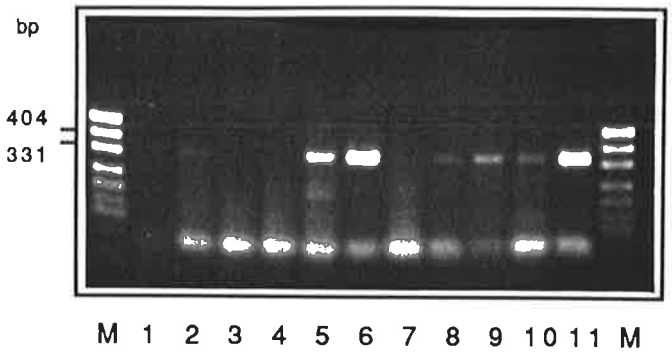
The identity of the 340 bp PCR product was confirmed by cloning this fragment into pGEM 5Zf+ and hybridising ^{32}P-labelled cDNA probes, prepared from this clone after excision of the primer region, to the GLRaV-3 clone, pGLR9ds.

8.3.3. Indexing of GLRaV-3 in four rootstocks and two "variable response" vines

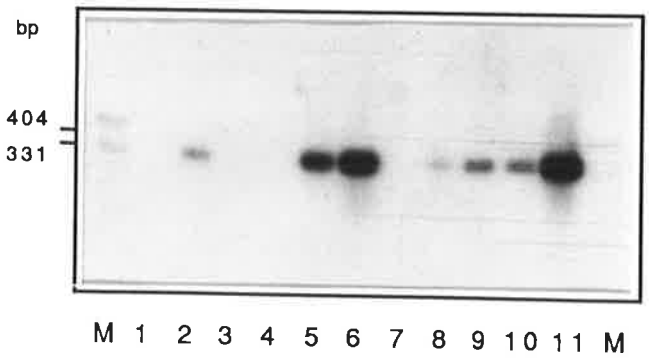
RT-PCR assay for GLRaV-3 was carried out on three Ramsey rootstocks, Black Malaga, CF-Frl and CF-Olz. The results (Fig. 8.1a and Table 8.2) showed that Ramsey 812 and Ramsey HR16 (lanes 3 and 4) were negative. Ramsey R1 (lane 2) and CS LC10 (lane 8) showed weak bands. CF-Frl and CF-Olz (lanes 9 and 10) gave stronger bands than CS LC10, while the Black Malaga TGR7 (lane 5) was clearly infected. The Tinta Carvahla and PN Antav 543 vines (lanes 6 and 11) gave strong bands at the position of the expected PCR product. A smear was present in Shiraz BVRC30 BV9 (lane 7). A repeat assay confirmed the above results and the absence of GLRaV-3 in Shiraz BVRC30 BV9. The positive results in CS LC10, CF-Frl and CF-Olz were consistent with the

Fig. 8.1: RT-PCR and Southern hybridization assay of GLRaV-3 in four rootstocks and two "variable response" vines. (**a**) RT-PCR assay was carried out on the vine RNA samples which were in **lanes 2-11**, Ramsey 1, Ramsey 812, Ramsey HR16, Black Malaga TGR7, PN Antav 543, Shiraz BVRC30 BV9, CS LC10, CF-Olz, CF-Frl and Tinta Carvahla respectively. In **lane 1**, was the negative control sample without added RNA. Amplification was carried out using the GLRaV-3 primer pair, T_a of 59°C and 27 PCR cycles. PCR products (in 8 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments. (**b**) Southern hybridization analysis was carried out on the PCR products using ^{32}P-labelled cDNA probes prepared by random priming. Autoradiography was carried out by exposure to Fuji RX film for 4 h.

(a)



(b)



leafroll symptoms observed on all three vines in the greenhouse at the end of the 1994 season.

The identity of the PCR products was confirmed by Southern hybridization analysis (Fig. 8.1b) using ^{32}P-labelled cDNA probes prepared from the GLRaV-3 clone, pGLR9ds.

When dot blot hybridization assay of the 10 vines was carried out using ^{32}P-labelled cDNA probes prepared by random priming, GLRaV-3 was detected only in Tinta Carvahla and PN Antav 543 (Table 8.2).

The results of indexing these 10 vines for GLRaV-3 by four diagnostic methods, bioassay, ELISA, dot blot hybridization assay and RT-PCR, are summarised in Table 8.2. Only Tinta Carvahla and PN Antav 543 were consistently positive for GLR by bioassay. GLRaV-3 was specifically detected by the other three methods. The RT-PCR assay was positive for five vines that were negative by hybridization assay, CF-Frl, CF-Olz, Black Malaga, Ramsey R1 and CS LC10. However, because the DNA sequences of the leafroll viruses of other serotypes were not yet known, possible homology of the GLRaV-3 primer sequence used in this study to the RNA sequences of the GLRaV of other serotypes could not be ruled out. Hence, co-infection of these five vines by the viruses of serotypes other than GLRaV-3 could possibly also account for the positive results observed.

8.3.4. Sensitivity of RT-PCR assay of GLRaV-3

The RT-PCR assay for GLRaV-3 was carried out on serial 10-fold dilutions of total RNA extract from PN Antav 543 (Fig. 8.2a). The expected PCR product was detected in a lower limit of 160 pg total RNA. In contrast, dot blot hybridization assay using ^{32}P-labelled cDNA probes detected GLRaV-3 in a lower limit of 400 ng total RNA (Fig. 8.2b). Thus, a 2,500-fold increase in the sensitivity of the RT-PCR assay was observed in comparison to the dot-blot hybridization assay.

Fig. 8.2: Comparison of sensitivity of detection of GLRaV-3 in PN Antav 543 by RT-PCR and dot blot hybridization assay. RNA extracts of PN Antav 543 (4 µg) were diluted 10-fold serially in 0.1 mM EDTA containing 10 ng/µl BSA (Bresatec, Australia). Aliquots of each dilution (4 µl) were used for RT-PCR and dot blot hybridization assay. **(a)** RT-PCR assay using the GLRaV-3 primer pair and the cycle parameters in Table 8.1. In **lanes 1-8**, 160 ng, 16ng, 1.6 ng, 160 pg, 16 pg, 1.6 pg, 160 fg and 16 fg total RNA were used respectively. In **lane 9**, was the negative control sample without added RNA. PCR products (in 5 µl) were separated on a 2% agarose gel. The DNA size markers were pUC 19/*Hpa*II fragments.
(b) Dot-blot hybridization assay using ^{32}P-labelled GLRaV-3 cDNA probes. Samples 1-6 contained 4 µg, 400 ng, 40 ng, 4 ng, 400 pg and 40 pg total RNA respectively.

Fig. 8.3: RT-PCR assay of GLRaV-1. Assay of GLRaV-1 was carried out using the putative GLRaV-1 primer pair and parameters in Table 8.1. The vine RNA samples in **lanes 3-8** were, PN Antav 543, CF-LR1, Emperor Vfr, Sultana 4BL, Ramsey 812, Ramsey HR16. In **lanes 2 and 9** respectively were, the negative control sample without added RNA and the positive control sample using CF-LR1-derived PCR products diluted 5000-fold. In **lanes 1 and 10** were the DNA size markers, pUC 19/*Hpa*II fragments. PCR products (in 5 µl) were separated on a 2% agarose gel.

(a)

(b)

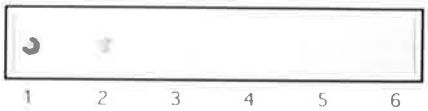


Table 8.2: Indexing of 10 vines for GLRaV-3 by four different diagnostic methods

Grape variety	Bioassay[a]	ELISA	DB	RT-PCR
Ramsey 1	-	-[b]	-	+
Ramsey 812	-	-[b]	-	-
Ramsey HR16	-	-[b]	-	-
Black Malaga TGR7	-	-[b]	-	+
PN Antav 543	+	+[c]	+	+
Shiraz BVRC30 BV9	nt	nt	-	-
CS LC10	-	nt	-	+
CF-Olz	V	nt	-	+
CF-Frl	V	nt	-	+
Tinta Carvahla	+	+[c]	+	+

nt, not tested.

DB, dot blot hybridization assay.

a, bioassay was carried out by green grafting of indicator varieties to rooted candidate vines at PISA, as described by Cirami et al. (1988).

b, c, ELISA was carried out using GLRaV-3 monoclonal antibodies (Bioreba) by N. Habili and A. Ewart respectively.

V, "variable response" shown by vines which do not produce regular leafroll symptoms in the field every year.

8.3.5. Application of RT-PCR assay to GLRaV-1

The RT-PCR assay for GLRaV-1 was developed using a CF-LR1 vine which indexed positive for GLRaV-1 by ELISA. The optimal cycle parameters for the putative primer pair for GLRaV-1 were derived as previously described (See 3.3.2). The best amplification was observed using a T_a of 58°C and 30 PCR cycles (Table 8.1).

The RT-PCR assay for GLRaV-1 was applied to six vine RNA samples, two Ramsey rootstocks, Ramsey 812 and Ramsey HR16, PN Antav 543, Emperor Vfr seedling and the two positive control vines, CF-LR1 and Sultana B4L (Fig. 8.3). The results confirmed the presence of GLRaV-1 in CF-LR1 and Sultana B4L (lanes 4 and 6). Ramsey 812, Ramsey HR16 (lanes 7 and 8) and the Emperor Vfr seedling (lane 5) were found to be negative. A weak band was observed in PN Antav 543 (lane 3), either as a result of mild infection by GLRaV-1 or of sequence homology of the GLRaV-1 primer sequence used in this study to the RNA sequences of the GLRaV of other serotypes as discussed in section 8.3.4.2.

Because of time restrictions, further work could not be carried out to confirm the GLRaV-1 sequences. However, the RT-PCR results provided additional evidence that cDNA clone pLR1 could possibly contain sequences of GLRaV1.

8.4. Discussion

A sensitive RT-PCR assay was developed for the detection of grapevine leafroll viruses, GLRaV-3 and possibly also for GLRaV-1, based on a modified RNA extraction protocol and the optimised RT-PCR protocol by fast cycle times developed for the diagnosis of grapevine viroids. A 2,500-fold higher sensitivity was achieved for the detection of GLRaV-3 by RT-PCR compared to dot blot hybridization assay. However, the assay of GLRaV by RT-PCR was less sensitive than that of grapevine viroids, most probably because of the presence of contaminants in the RNA extract, as indicated by the A_{260}/A_{230} ratio (1.33 for the viral RNA extracts as compared with 1.9 for the viroid RNA extracts).

The sensitivity of the RT-PCR assay for GLRaV-3, obtained with RNA extracts prepared by this modified protocol, was found to be at least 1000-fold more sensitive than that obtained with RNA prepared by "fast extraction" protocols, e.g., using the Gene Releaser™ polymeric matrix (Levy et al., 1994) and the rapid virus release protocol (Thomson and Dietzgen, 1995) (data not shown). Thus, although the presence of pathogens was detectable by these two "fast extraction" protocols, which could therefore have practical applications in preliminary screening procedures for large scale indexing, the ultimate sensitivity of pathogen detection was observed still to require RNA extracts of the highest quality.

Because of the presence of many grapevine viruses in the phloem tissues (Martelli, 1993), this RT-PCR assay and extraction protocol should be applicable to the diagnosis of numerous viral diseases of grapevines, provided that adequate sequence information is available for the design of the PCR primers.

Chapter Nine

General Discussion

9.1. Sensitivity of diagnostic methods and viroid detection

The present study on viroids in grapevines, which involved two of the most sensitive methods available for viroid detection, dot blot hybridization assay with ^{32}P-labelled riboprobes and RT-PCR, highlighted the critical importance of the quality of RNA extracted from plants to the sensitivity of the diagnostic methods.

The detection of low titre viroids in grapevine tissue was most likely aided by the high efficiency of the viroid RNA extraction procedure developed specifically for RT-PCR analysis. Both AGV and CEV, previously reported to require passage through alternative hosts to increase their titre before detection (Rezaian et al., 1988), were detected directly in vineyard-grown vine leaf samples. In addition, GYSV1, AGV and HSV were detected in the RNA extracts of SAMC-derived vines (See Table 4.2) and in grape seedlings (See Table 6.1), both of which have generally been considered as viroid-free.

The greater sensitivity of the RT-PCR assay by the optimised protocol for fast cycle times, compared to the dot blot hybridization assay, was shown by the 25,000-fold increase in sensitivity observed for the detection of GYSV1 in a high GYSV1 titre sample. Hence, it was not surprising that different viroid profiles (See 3.3.3) were found in grapevines according to the viroid detection technique used (See Table 9.1). Viroids, beyond the limit of detection by dot blot hybridization assay were detected in vine RNA extracts by RT-PCR: CEV was detected by RT-PCR in the 10 field-grown vine samples and HSV in seedlings and in vines regenerated by SAMC.

However, in spite of the higher sensitivity of the optimised RT-PCR protocol for fast cycle times as compared to the standard RT-PCR protocol, the limit of this technique was reached in the detection of viroids in grape seedlings and SAMC-derived vines. A higher level of sensitivity was required for the RT-PCR assay of both GYSV1 and HSV in these latter vines and both viroids were only amplified in the presence of TaqStart™ Antibody to prevent or limit the amplification of non-specific PCR products. The transmission of HSV to grape seedlings and SAMC-derived vines was demonstrated only by this improved RT-PCR assay (See 6.3.3.2 and 7.3.5) and no other technique.

Table 9.1. Viroid profiles in grapevines as assayed by dot blot hybridization and RT-PCR

Viroid	Source of vines / Assay technique					
	Vineyard[a]		SAMC[b]		Seedling[c]	
	DB	RT-PCR[d]	DB	RT-PCR[e]	DB	RT-PCR[e]
GYSV1	+	+	+	+	+	+
GYSV2	nt	+	nt	nt	nt	nt
HSV	+	+	-	+	-	+
AGV	+	+	+	nt	+	nt
CEV	-	+	-	nt	-	nt

nt, not tested.

a, viroid profile obtained from 10 grapevine varieties grown in the vineyard (Table 3.2).

b, viroid profile typical for SAMC-derived vines (See 4.3.4, Table 4.2 and Table 7.3).

c, viroid profile typical for seedlings (See 6.3.5 and Table 6.1).

d, RT-PCR assay was carried out using the optimised protocol for fast cycle times (See 3.2.6.2).

e, RT-PCR assay was carried out using the optimised protocol for fast cycle times and TaqStart™ Antibody (See 6.3.3.2).

DB, dot blot hybridization assay using ^{32}P-labelled riboprobes (See 3.2.12).

9.2. Sequence variation in grapevine viroids

The presumed influence of sequence variation on the behaviour of viroids was observed throughout this study: in the amplification of viroids by RT-PCR, in the viroid population of SAMC-derived vines, in seedlings and in micropropagated vines.

This influence was found to decrease the sensitivity of detection of GYSV1 by RT-PCR even when PCR primers, designed in the conserved domains, were used to minimise such an influence. Although Rigden and Rezaian (1993) found sequence variation mostly in the P domain, Polivka et al. (1996) observed sequence variation even in the inverted repeat sequences bordering the central conserved domain. Altered thermodynamic stability or secondary structure as a result of base substitutions in the PCR primer region could possibly account for the altered efficiency in amplification. However, this particular problem could be limited to GYSV1, because HSV and GYSV2 were not observed to have the same high level of sequence variation (Rigden and Rezaian, 1993; Polivka et al., 1996).

The presence of a population of GYSV1, HSV and AGV, that did not build up to high titres in SAMC-derived vines (See 4.3.3) and in seedlings (See 6.3.5), suggested a selective transmission mechanism involving specific viroid variants. Evidence for the transmission of such variants was provided by the detection of a single GYSV1 band at the same mobility in seven seedlings of four grape varieties by Northern hybridization analysis of RNA extracts after separation on a 5% denaturing PAGE (See 6.3.2.1). The position of this GYSV1 band associated with seedlings was different from that of the faster moving GYSV1 bands (circular and linear) of the stock vine from which one of the seedlings was derived.

The presumed influence of viroid variants was also observed in the differential increase in viroid content observed in micropropagated vines. Differences in the P domain of naturally-occurring sequence variants of CEV (Visvader and Symons, 1986) and PSTV (Schnölzer et al., 1985) have been correlated with the severity of symptoms

produced by these viroids as a consequence of the different titres attained by the various viroid variants (Visvader and Symons, 1986; Diener et al., 1993).

Since this study was concerned mainly with the detection of viroids in low titre in grapevine tissue and the behaviour of viroids under different culture or treatment conditions, sequencing studies were not carried out to further characterise the interesting viroid variants which have been observed in a number of instances. Hence, large scale cloning and sequencing of the viroid variants reported in the different chapters would be a logical extension of the work carried out in this study to confirm the results reported here. This exercise would be facilitated by the preliminary amplification by RT-PCR of these variants, normally difficult to isolate directly from grapevine tissues because of their low titre, using the protocols developed in the course of this study.

9.3. AGV in grapevines

This study revealed that AGV, which has been little studied in most of the work reported so far on grapevine viroids, is much more widespread than previously reported (Rezaian et al., 1992). The presence of AGV in the 10 vineyard-grown vine varieties tested, in the stock and progeny vines indexed in the micropropagation experiment and its persistence in SAMC-derived vines, in seedlings and in cold-treated stock vines, contrasts with its previous detection in only one out of 12 Californian grapevines by Northern hybridization analysis using ^{32}P-labelled cDNA transcripts of M13 AGV clones as probes (Rezaian et al., 1992). The transmission of AGV to seeds and meristems closely resembled that of GYSV1 and provided invaluable confirmation of the results obtained on GYSV1. Its widespread distribution in grapevines as well as its easy detection by dot blot hybridization analysis qualifies AGV as an interesting grapevine viroid for future studies.

9.4. Biological significance of viroids in grapevines

One of the aims of the work described in this thesis was to produce viroid-free vines as a means to evaluate the biological significance of viroids in grapevines. This aim

was not achieved in spite of efforts to produce SAMC-derived vines from selected, viroid-indexed stock vines in conditions designed to favour viroid elimination. The persistence of GYSV1 and HSV in vines regenerated from meristems under carefully controlled conditions reinforced the intriguing question of the role of viroids in grapevines (Semancik et al., 1993).

The use of grape seedlings and SAMC-derived vines as "viroid-free" vines was reported by many workers in experiments involving the re-introduction of viroids into putative viroid-free vines (See 1.7.6) to assess the significance of viroids to grapevine performance. Although this present study provided strong supporting evidence for the presence of viroids in these two types of vine tissue, subsequent changes in vine performance as a result of viroid re-introduction could perhaps still be attributed to an influence of the viroids inoculated because the viroid population in seedlings and SAMC-derived vines has been observed to remain stable at a low level. However, the fact remains that a viroid-free background does not appear to be obtainable and any experimentation involving the re-introduction of viroids would, in fact, be evaluating the effect of different viroid levels as well as of different sequence variants on grapevine performance rather than the biological significance of viroids in grapevines.

Such studies could be extended by the setting up of an additional replicate set of re-inoculated vines, grown under conditions of high temperature and illumination similar to those provided in micropropagation conditions to induce the viroids to high titres before transfer to the field, in order to extend the range of viroid levels in vines with which to study the effect of viroids on vine performance.

The indexing of Fantasy Seedless, obtained originally from the Foundation Plant Materials Service, California, showed a viroid profile similar to that observed only in seedlings and SAMC-derived vines (See 5.3.2.2). This grapevine selection, which had originally tested negative to all known viruses, would be an excellent candidate for future

re-inoculation experiments under field conditions, since previous experiments of this type were prematurely terminated by phylloxera infection (Wolpert et al., 1996).

9.5. Future work

The work reported in this thesis opens up a range of possibilities for future studies in several areas that are briefly summarized below. Analysis of the sequences of the viroid variants that have been discussed above (See 9.2) could perhaps provide a better understanding of the role of sequence variation in viroid biology and may shed some light on the mechanism of transmission of viroids to seeds and meristems.

(a) Future cloning and sequencing studies would include the viroid sequences or viroid populations observed in the vines specified:

-the GYSVI population in the Ramsey rootstock (See 3.4.3) which had a low efficiency of amplification by RT-PCR, an observation inconsistent with the high GYSV1 content observed by dot blot hybridization analysis.

-the AGV population in Ramsey R8 (See 4.3.1.2) which showed an exceptional increase in AGV titre in the presence of high levels of GYSV1 and HSV.

-the higher molecular weight GYSV1 bands (See 6.3.3.2) observed in the amplification of GYSV1 in Sultana H5 by the c4h4 GYSV1 primer pair to verify the possibility of end repeats in the T1 domain of GYSV1.

-the PCR products amplified from GYSV1 in the RTG, Vfr, PN1, PN2, Ries, Sem1 and Sem2 seedlings (See 6.3.3.2) and their respective stock vines to confirm the transmission of specific GYSV1 variants in seedlings.

-the GYSV1 and HSV population in Sultana H5 and Shiraz BVRC30/BV8, stock and micropropagated vines (Table 5.1), to confirm the change in the structure of viroid populations as a result of micropropagation.

(b) The possibility of end repeats in GYSV1 can be further investigated by the amplification by RT-PCR of the T2 region of GYSV1 and the cloning of eventual high molecular weight fragments. This RT-PCR-based approach would be the simplest way to resolve this issue because the putative higher molecular forms of GYSV1 were in extremely low amounts and were observed only after autoradiographic exposure for 1 week.

(c) The relative contribution of GYSV1 and GYSV2 to the development of the grapevine yellow speckle disease can be studied using the dot blot hybridization and RT-PCR assays that enable the specific discrimination between these two viroids.

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APPENDIX

Part of the work described in this thesis has been submitted for publication to the Journal of Virological Methods:

Wan Chow Wah, Y.F. and Symons, R.H. (1996) A high sensitivity RT-PCR assay for the diagnosis of viroids in grapevines in the field and in tissue culture.