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Phytoplasmas in Australian grapevines - detection, differentiation and associated diseases

by

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S u m m a r y: Phytoplasmas associated with Australian grapevine yellows (AGY) symptoms were detected using the polymerase chain reaction (PCR). To optimise the diagnostic, nested PCRs were compared with single PCRs using different primer pairs. Grapevine DNA known to be AGY phytoplasma positive was serially diluted and subjected to nested and single round PCR tests to determine which was the most sensitive. Samples taken over two growing seasons were used to determine the optimum sampling time for phytoplasma detection. The specificity of primer pairs was determined using phytoplasmas detected in Australian grapevines and overseas reference grapevine phytoplasmas. DNA extracted from grapevine exhibiting a range of symptoms was screened for phytoplasmas. Two different phytoplasmas were amplified in the PCR and they were identified using specific PCR primers and by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene and 16S rRNA/23S rRNA spacer region. RFLP analysis confirmed that one phytoplasma was the AGY phytoplasma and the other phytoplasma was indistinguishable from the tomato big bud (TBB) phytoplasma. The AGY phytoplasma was associated with AGY symptoms but was occasionally detected in asymptomatic vines and those with late season leaf curl (LSLC) and restricted growth (RG) symptoms. The TBB phytoplasma was detected in some vines with LSLC symptoms and very occasionally in vines with AGY symptoms. A 'variant' of the AGY phytoplasma was also detected in vines showing typical AGY symptoms.

K e y w o r d s : phytoplasma, Australian grapevine yellows, nested PCRs, RFLP.

Introduction

Australian grapevine yellows (AGY) is a disease of grapevines first reported in 1975 (MAGAREY and WACHTEL 1983). A phytoplasma etiology was suspected (MAGAREY and WACHTEL 1986) and confirmed more recently using molecular diagnostic techniques (PADOVAN *et al.* 1995). The disease is characterised by yellowing, downward curling of leaves on stunted shoots that do not harden off but remain rubbery (MAGAREY and WACHTEL 1985). Shoot tips die and bunches shrivel and fall (MAGAREY and WACHTEL 1986). The disease appears most often in Chardonnay and Riesling, but has also been reported in other cultivars. The disease is now present in nearly every viticultural district of Australia (MAGAREY and WACHTEL 1986).

AGY is similar to other grapevine yellows diseases and a summary of these diseases occurring worldwide, and the identities of their associated phytoplasmas, is given in PADOVAN *et al.* (1995). Based on 16S rRNA gene sequence data, PADOVAN *et al.* (1996) reported that the AGY phytoplasma is unique but most closely related to the phytoplasmas associated with grapevine diseases in the stolbur group. DAVIS *et al.* (1997) also showed that while the AGY phytoplasma was closely related to the European stolbur phytoplasma, it was unique and represented a new taxon, "Candidatus Phytoplasma australiense". LIEFTING *et al.* (1998) compared 16S rRNA gene and spacer region sequences and proposed that, along with AGY, the phytoplasmas associated with Phormium yellow leaf (PYL) and papaya dieback (PDB), should also be considered as "Candidatus Phytoplasma australiense".

Recent studies in Australia showed that, in addition to AGY, disorders referred to as late season leaf curl (LSLC) and restricted growth (RG) occur on grapevine and some of the vines had a combination of these diseases (CONSTABLE *et al.* 1998). The aim of this study was to optimise the diagnostic for phytoplasmas in grapevines and to increase our understanding of the role of phytoplasmas in the grapevine diseases AGY, LSLC and RG. In addition, attention was paid to vines showing combinations of these disorders to provide more evidence that vines can express symptoms of more than one disorder at the same time. These vines were screened for phytoplasmas to increase our understanding of the diversity of phytoplasmas infecting Australian grapevines.

Materials and Methods

Source of phytoplasmas: Grapevines without symptoms and those with symptoms of AGY, LSLC and RG were collected from plantings at three vineyards in the Sunraysia district; Karadoc in Victoria, Gol Gol in New South Wales, and Wenem in Victoria. The plantings were 4-5 years old Chardonnay on a variety of rootstocks (at Karadoc), on their own roots (at Gol Gol), and on Ramsey and Schwarzmann (at Wemen). Chardonnay grapevines with symptoms of AGY were also collected from the Ovens Valley alpine region of Victoria. Sweet potato with sweet potato little leaf (SPLL) disease was collected near Darwin, Northern Territory in 1991 and tomato with TBB disease was collected near Adelaide, South Australia in 1992. Additional phyto-

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plasmas which have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequence (SCHNEIDER *et al.* 1993; SEEMÜLLER *et al.* 1994) were included as reference strains. The sources of Molière's disease of cherry (MOL) from France, stolbur of Lycopersicon esculentum (STOLF) from France, American aster yellows (AAY) from Florida and sunn hemp or Crotalaria witches' broom (SUNHP) from Thailand are as described previously (SCHNEIDER *et al.* 1993). All reference phytoplasmas were transmitted to and maintained in periwinkle.

Extraction of DNA: DNA was isolated from the leaves, petioles and young stems of field-collected plants and the reference strains using a phytoplasma enrichment procedure (AHRENS and SEEMÜLLER 1992). The nucleic acid pellet was resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and 3 µl of the sample was subjected to electrophoresis in a 1 % agarose gel using 0.5 TBE as running buffer. Products in gels were stained with ethidium bromide and then visualised by UV transillumination. The quality and quantity of the DNA was estimated from the gel and the nucleic acid was used as a template for PCR either undiluted or after 1/10 dilution.

Polymerase chain reaction (PCR) to detect phytoplasmas: For PCR, each reaction contained 0.2 mM of each dNTP, 0.4 mM of each primer, 1 DNA polymerase buffer supplied with the enzyme and 0.2 U thermostable DNA polymerase (Advanced Biotechnologies Ltd, Surrey, U.K.). For first round or single PCR, 1 ml of undiluted or 1/10 diluted nucleic acid sample was added to the PCR cocktail mix. For nested PCR, 1 ml of the first round reaction mix was added to the PCR cocktail mix containing the second primer pair. The total reaction volume was 50 ml in a Corbett FTS-320 thermocycler (Corbett Research, Mortlake, NSW, Australia). For first round nested PCR with primer pair P1/P7, a manual hot start PCR at 92 °C for one minute was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. PCR conditions for second round nested PCR with primer pairs R16F2/m23sr, and PCR with primer pair fStol/rStol were the same except that the annealing temperature was 58 °C. Similarly, PCR with primer pairs P1/AGY 2 and fStol/AGY 2 was the same except that the annealing temperature was 53 °C . After amplification, 5µl from each sample was subjected to electrophoresis in a 1.0 % agarose gel and the DNA was visualized by staining with ethidium bromide and UV illumination. Total nucleic acid extracted from asymptomatic plants were subjected to the PCR as a negative control and in some experiments water controls were included, in which no plant nucleic acid was added to the PCR reaction mix.

PCR primers used to detect phytoplasmas in grapevine are listed in Tab. 1. For the nested PCR, the primer pair P1 and P7 were used, followed by the primer pair R16F2 and m23sr. These primers are specific for a region of the 16S rDNA gene in all known phytoplasmas. A single PCR with the primers f/rStol was used to amplify a region of the 16S rDNA gene of the European grapevine yellows phytoplasmas. To develop a diagnostic assay that was specific for AGY, the primer pair P1 and AGY 2 was used to amplify a region of the 16S rRNA and 16S-23S spacer region (SR). This potentially AGY-specific test was compared with another test using the primer pair fStol and AGY 2 which also amplified a region of the 16S rRNA and 16S-23S SR. AGY 2 was designed as a result of comparisons of the 16S-23S SR sequences of the AGY phytoplasma with sequences of other phytoplasmas (GIBB *et al.* 1998).

RFLP was used to differentiate phytoplasmas. 5 µl of the PCR products amplified in the nested PCR were digested using the restriction enzymes *Alu*I, *Rsa*I, *Mse*I and *Hpa*II in the buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA). Digestions were incubated overnight at 37 °C and the fragments were separated by electrophoresis in an 8 % polyacrylamide gel in 1 TBE buffer and visualised by staining with ethidium bromide and photographed on a UV transilluminator.

Optimising the diagnostic test: DNA from two phytoplasma positive grapevine samples was quantified using a DNA Mass Ladder (Life Technologies Pty Ltd, Vic., Australia). To compare the sensitivity of the diagnostic tests, the DNA was then diluted and subjected to four different PCR tests using either the nested primers, f/rStol, P1/AGY2 or fStol/AGY2.

Optimising the sampling time for phytoplasma detection: Twenty grapevines growing in the commercial vineyard at Gol Gol were tagged in October 1995 and samples were taken monthly from October through May over two years. Most of the grapevines were chosen at random because it was too early to observe symptoms of AGY disease. Some of the vines chosen appeared slow in their new season's growth. The shoot, petiole and leaf samples were tested for phytoplasmas by PCR using both the nested PCR with primers P1/P7 and R16F2/m23sr, and single round PCR using primers fStol/AGY2.

Phytoplasma diversity: To obtain more information about the identity of the new phytoplasma in Australian grapevine, PCR products amplified by the nested PCR were subjected to RFLP analysis using the restriction enzymes, *Alu*I, *Rsa*I and *Mse*I. The new phytoplasma was compared to AGY and representatives of most of the major phytoplasma groups. During the course of this study, grapevine samples showing AGY symptoms were collected from the Ovens Valley region in Victoria. Samples with AGY symptoms which tested positive in the nested PCR, were subjected to RFLP analysis using the restriction enzymes *Alu*I, *Hpa*II, *Mse*I and *Rsa*I.

Correlation between symptoms and presence of phytoplasma: Seasonal field studies and a recent report indicated that grapevines exhibited a range of symptoms and often a single grapevine was observed with more than one symptom. These observations highlighted a need for tests that could be used to detect and differentiate phytoplasmas in grapevine. At this stage, specific phytoplasmas were not definitively linked to specific diseases so as a first step towards understanding the role of phytoplasmas, samples taken from vines with a range of disorders were tested using both universal and AGY specific tests. Vines with different symptoms chosen on the

Table 1

Primer sequences used in PCR tests

Primer	Sequence 5' - 3'	Reference
P1	AAG AGT TTG ATC CTG GCT CAG GAT T	Deng & Hiruki 1991
P7	CGT CCT TCA TCG GCT CTT	Kirkpatrick *et al.* 1994
R16F2	ACG ACT GCT AAG ACT GG	Lee *et al.* 1993
m23sr	TAG TGC CAA GGC ATC CAC TGT G	Padovan *et al.* 1995
fStol	GCC ATC ATT AAG TTG GGG A	Maixner *et al.* 1995
rStol	AGA TGT GAC CTA TTT TGG TGG	Maixner *et al.* 1995
AGY2	GAT GTG ACC TAT TTT ATT TG	Gibb *et al.* 1998

basis of having been positive for phytoplasma using the universal nested PCR test, were subjected to the AGY specific test. The reference phytoplasmas, TBB and the AGY phytoplasma were included in these tests. To test the AGY specific PCR against European grapevine phytoplasmas, DNA extracted from the grapevine with LSLC containing the phytoplasma amplified by nested PCR, the AGY phytoplasma, and grapevine phytoplasma DNA from Germany (VK) and France (BN), were subjected to PCR using either the nested primers, f/rStol, fStol/AGY2 or P1/AGY2.

Between 1995 and 1998, a comprehensive screening program was completed in which grapevines with a range of symptoms including AGY, LSLC, RG, or a combination of these diseases including AGY/RG, AGY/LSLC, AGY/RG/LSLC, RG/LSLC and asymptomatic grapevine, were tested for phytoplasma.

Results

Optimising the diagnostic test: When testing DNA from one phytoplasma positive grapevine sample, the nested PCR amplified products down to 0.04 ng total plant DNA but the result was inconsistent in that no product was amplified when 0.08 ng DNA was used and 2 ng appeared to inhibit the reaction (Fig. 1). For the second sample, detection was down to 0.08 ng DNA. The f/rStol primers amplified phytoplasma DNA at all concentrations of total plant DNA and P1/AGY2 was the least sensitive in that detection was only down to 0.4 ng DNA with possible inhibition at higher concentrations. The fStol/AGY2 primers permitted detection down to 0.08 ng DNA.

Optimising the sampling time for phytoplasma detection: Sampling started in October and at that time it was too early to observe symptoms of AGY in the field, but some of these vines were slow in their new season's growth. Others appeared to be asymptomatic with no unusual growth. At this stage the identity of phytoplasmas at the study site was not known so a universal phytoplasma detection test was considered the most appropriate. Based on PCR results, phytoplasma DNA was detected from most plants when samples were taken in January and February (Tab. 2). For the first year, the two PCR tests were compared at each sample time and at the optimum sample time in early February, the same number of samples were found to be positive by both tests

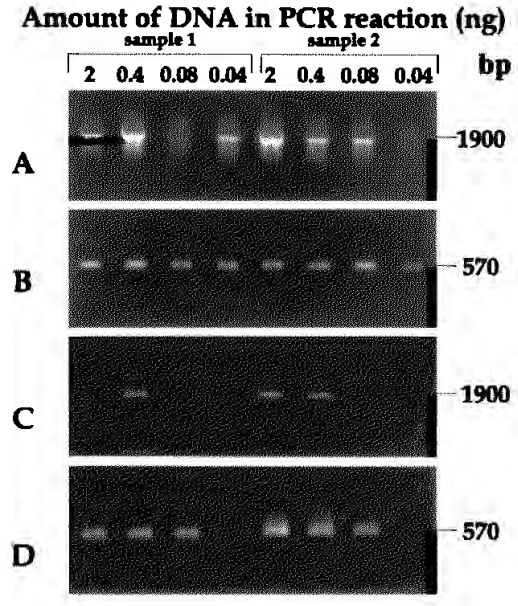

Fig. 1: Testing PCR detection limits for 4 different primer sets. Two samples of total DNA from phytoplasma positive samples were diluted and subjected to nested PCR using the primers P1/P7; R16F2/m23sr (**A**), single round PCR using f/rStol (**B**); P1/AGY2 (**C**) and fStol/AGY2 (**D**). DNA markers are not shown but DNA band sizes are indicated in base pairs.

Table 2

The same 20 grapevines were tested over two years for phytoplasma by PCR to determine the best sampling time for phytoplasma detection

Month sampled	Number positive by nested PCR/20	
	1995/96	1996/97
Oct	NT	1
Nov	NT	9
Dec	0 (0)[A]	9
Jan	10(6)	15
Feb	14(14)	7
Mar	6 (1)	0
Apr	0 (0)	0
May	NT	0

[A]Numbers in parentheses refer to samples amplified by single PCR using the primers fStol/AGY2. NT = not tested.

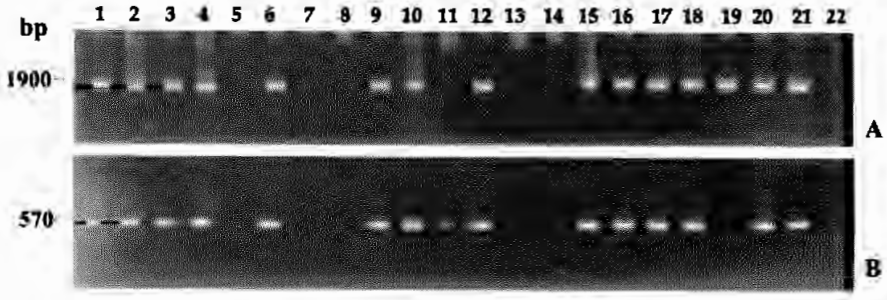

Fig. 2: Amplification of phytoplasma DNA from grapevine by nested PCR using the primers P1/P7; R16F2/m23sr, which amplifies a region of the 16S rRNA gene and the spacer region between the 16S rRNA and 23S rRNA genes (**A**) and by single PCR using the stolbur specific primers f/rStol (**B**). Lanes 1-20 = cohort of 20 vines used for monthly samplings over two growing seasons with samples taken February 1996 for the PCR results shown here, lane 21 = AGY phytoplasma positive control, lane 22 = water negative control. DNA markers are not shown but DNA band sizes are indicated in base pairs.

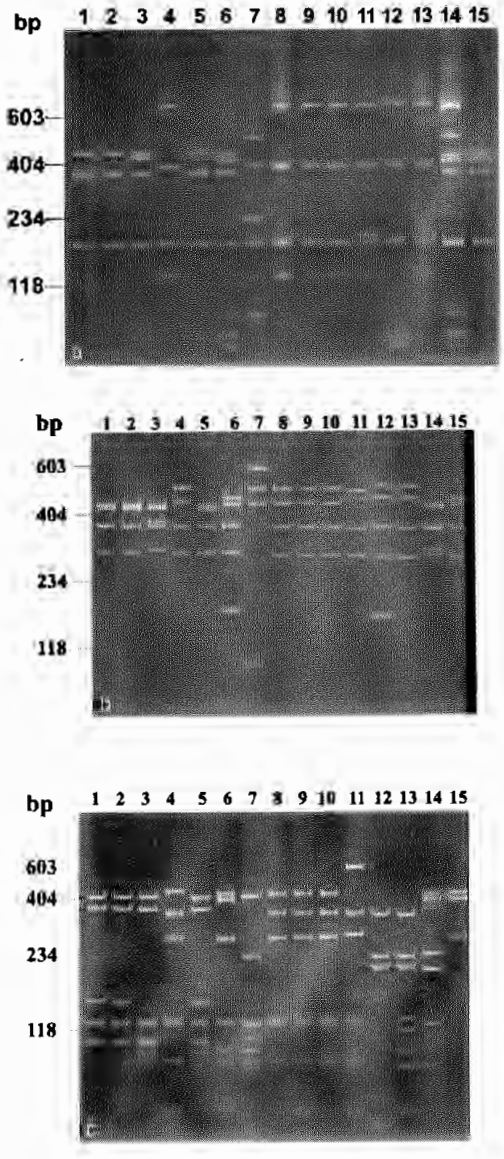

Fig. 3: RFLP analysis of PCR products amplified by the primers P1/P7;R16F2/m23sr. Lane 1 is Vergilbungskrankheit (VK) from Germany; lane 2 is boir noir (BN) from France; lane 3 is the AGY phytoplasma; lane 4 is GP-A; lane 5 is stolbur (STOLF) of *Lycopersicon esculentum* from France; lane 6 is American aster yellows (AAY) from Florida; lane 7 is apple proliferation (AP) from Germany; lane 8 is sunn hemp or Crotalaria witches' broom (SUNHP) from Thailand; lane 9 is sesame phyllody (SEPT) from Thailand; lane 10 is phyllody of *Cleome viscosa* (CLP) from Thailand; lane 11 is Crotalaria phyllody (CROP) from Thailand; lane 12 is witches' broom of *Vaccinium myrtillus* (VAC) from Germany; lane 13 is green valley X strain in periwinkle from the USA; lane 14 is Blütenverkleinerung (BVK) from Germany and lane 15 is brinjal little leaf (BLL) from India. PCR products were digested with *Alu*I (**a**), *Rsa*I (**b**) and *Mse*I (**c**). DNA markers are not shown but are indicated by the sizes given in base pairs.

(Fig. 2). Except for two samples, the same vines were found to be positive for phytoplasma. The only advantage in using the nested PCR test was that later in the season when symptoms were less obvious, phytoplasma could be detected in more vines (Tab. 2). For the first year, of the 14 grapevines found to be positive, 12 were also positive on other dates and of these, three were not positive on consecutive sampling dates. Two of the 14 grapevines were only positive on the optimum sample date. One sample was negative on the optimum sample date but positive on another date. There was good correlation between the test and symptoms, and in February 1996 for example, of the 14 phytoplasma positive vines, 13 had symptoms of AGY disease and one was asymptomatic. Of the 6 phytoplasma negative vines, all were asymptomatic.

Phytoplasma diversity by RFLP analysis: Using the restriction enzymes, *Alu*I, *Rsa*I and *Mse*I, DNA patterns confirmed that AGY is closely related but not identical to VK and BN (Fig. 3). A grapevine with LSLC symptoms which tested positive in the universal phytoplasma test was considered infected with a grapevine phytoplasma from Australia (GP-A). For all three restriction enzymes, GP-A gave a pattern identical to SUNH, a phytoplasma of *Crotalaria juncea* from Thailand which is also similar to the Australian tomato big bud (TBB) phytoplasma.

Following DNA digestion with *Alu*I, the Ovens Valley AGY phytoplasma was very similar to the AGY phytoplasma with only minor changes in band sizes (Fig. 4). When DNA was subjected to digestion using *Hpa*II and *Mse*I, differences between the AGY and the Ovens Valley AGY phytoplasma DNA were more marked. With *Rsa*I, some differences were also observed in band sizes. With all enzymes, the two Ovens Valley AGY phytoplasma samples were identical.

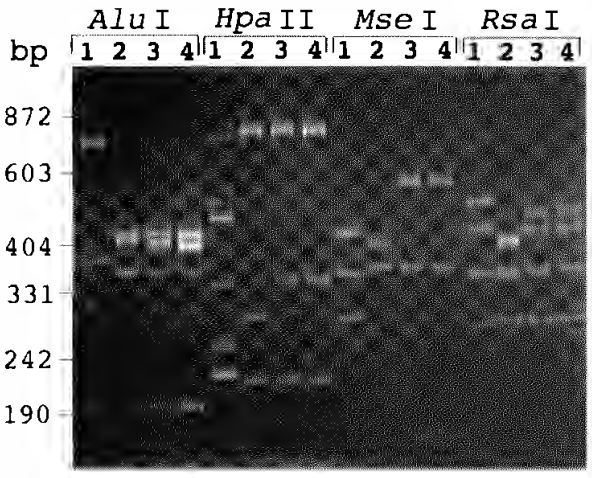

Fig. 4: RFLP analysis of PCR products amplified by the primers P1/P7;R16F2/m23sr. Restriction enzymes are indicated on the gel and for each enzyme, 1 is TBB, 2 is AGY and 3-4 are two examples of 'AGY variants'. DNA markers are not shown but are indicated by the sizes given in base pairs.

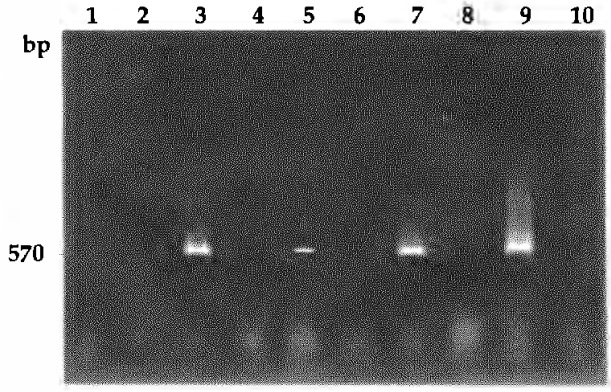

Fig. 5: PCR screening of grapevine DNA shown to be phytoplasma positive by nested PCR, using the AGY specific primers fStol/AGY2. Grapevine samples tested showed a range of symptoms i.e. lane 1-2 AGY and LSLC; lane 3 AGY.; lane 4 AGY, LSLC, RG; lane 5 AGY, LSLC; lane 6 LSLC; lane 7 AGY; lane 8 is a TBB phytoplasma positive control; lane 9 is an AGY phytoplasma positive control and lane 10 is a water negative control. DNA markers are not shown but DNA band size is indicated in base pairs.

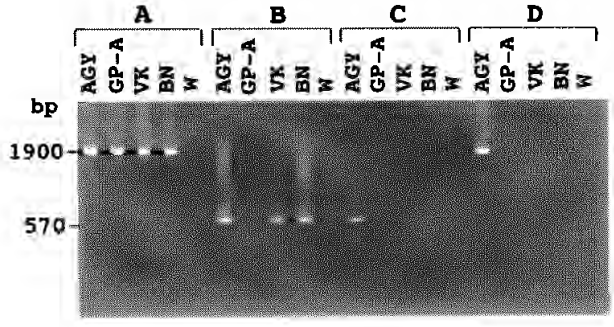

Fig. 6: PCR amplification of phytoplasma DNA from grapevine with LSLC symptoms and shown to contain a phytoplasma (GP-A) not amplified by the AGY specific primers. Reference samples were the AGY phytoplasma, the grapevine yellows phytoplasmas Vergilbungskrankheit (VK) from Germany and bois noir (BN) from France. Primer combinations were (**A**) P1/P7;R16F2/m23sr, (**B**) f/rStol, (**C**) fStol/AGY2 and (**D**) P1/AGY2. DNA markers are not shown but DNA band sizes are indicated in base pairs.

Correlation between symptoms and presence of phytoplasma: On the basis of the sensitivity tests it was decided that an AGY specific PCR using the primers fStol/AGY2 was better than P1/AGY2. Using the primer pair fStol/AGY2, the TBB reference phytoplasma was not amplified but the AGY reference sample was, giving a band of approximately 570 bp (Fig. 5). Grapevines expressing a range of symptoms and which had tested positive in the universal phytoplasma test, were subjected to PCR using the AGY specific primers. Two grapevine samples showing only AGY symptoms were positive. Three grapevines with symptoms of LSLC throughout the entire plant and with AGY limited to one or two spurs, were also tested. Of these, two were negative and one was positive. One grapevine with RG and LSLC over the entire vine also had AGY symptoms confined to one or two spurs. This vine also tested negative. A grapevine with LSLC symptoms tested negative and the asymptomatic grapevine sample was also negative.

In tests on European grapevine phytoplasmas, all samples tested positive using the universal nested PCR primers (Fig. 6). Using f/rStol which was designed to amplify the stolbur grapevine phytoplasmas, VK, BN and AGY but not GP-A were amplified. The AGY specific tests using fStol/AGY2 and P1/AGY2 were indeed AGY specific and neither VK, BN nor the GP-A phytoplasma were amplified.

In the two year phytoplasma survey, 60 % of grapevines exhibiting only AGY symptoms or AGY symptoms in combination with RG, were phytoplasma positive using the universal phytoplasma test. The vast majority of these were the AGY phytoplasma (Tab. 3). Of the 35 grapevines with symptoms of AGY and LSLC, only 26 % were phytoplasma positive, but this is significantly greater than for asymptomatic vines (3 %). Where vines had AGY and LSLC symptoms, half of the phytoplasmas detected were the AGY type and the other half were the TBB type. Eleven vines with all three disorders were tested, but of these, 46 % were phytoplasma positive with an almost equal split between AGY and TBB phytoplasma types. For vines with LSLC symptoms only, 10 % were phytoplasma positive with twice as many being TBB than AGY phytoplasma type. Of the 203 vines with symptoms of RG tested, only 5 % were phytoplasma positive and this phytoplasma was the AGY type. Of the 28 vines with symptoms of RG and LSLC, 4 % were phytoplasma positive and the phytoplasma was the TBB type. The results for vines with symptoms of RG and RG/LSLC are similar to those for asymptomatic vines in which 3 % tested phytoplasma positive.

Discussion

When total DNA which was known to be phytoplasma positive, was diluted and tested in the PCR, the nested PCR was not more sensitive than the single round PCRs. In fact, the single PCR using primers specific for the stolbur phytoplasmas was more sensitive. This was less useful however than universal screening when testing grapevines with a

Table 3

Grapevines with different symptoms were tested throughout 1995/96 and 1997/98 for phytoplasma. The number positive by PCR and the type of phytoplasma detected were recorded

Symptoms	Plants tested	Positive	AGY	TBB	AGY variant	AGY & TBB	Not known
AGY	223	131 (59 %)	94	5	6	1	25
LSLC	126	12 (10 %)	4	8			
RG	203	11 (5 %)	9				2
AGY/RG	66	40 (61 %)	30	3	1		6
AGY/LSLC	35	9 (26 %)	4	4			1
AGY/RG/LSLC	11	5 (46 %)	3	2			
RG/LSLC	28	1 (4 %)		1			
Asymptomatic	381	10 (3 %)	5	1			4

range of symptoms that may have been associated with, as yet, unidentified phytoplasmas. When using the AGY specific tests the universal forward primer paired with the AGY primer resulted in a PCR that was less sensitive than the PCR using the forward stolbur specific primer and AGY specific reverse primer. These sensitivity tests allowed us to choose the best AGY phytoplasma specific test but also indicated that the other primer combinations may be useful in PCR tests aimed at differential detection of phytoplasmas, especially if more than one was detected by universal nested PCR in field collected vines.

During the course of this study there were problems with PCR detection of phytoplasmas in grapevine. This problem had been noted in previous studies where we found that grapevines sampled early in the season were phytoplasma negative, mid season they were positive and later in the season they were again negative. These problems needed to be addressed if we are to use PCR as a routine tool to further our understanding of the association between the AGY disease and the phytoplasma. To address the issue of optimum sampling time for detection, the same set of grapevines were sampled at different times throughout the growing season and the ability to detect phytoplasmas was determined. Results showed that there was an increase in the number of phytoplasma positive grapevines reaching a maximum early January to early February but detection fell away dramatically after this. The different result for the same vine between years is possibly a reflection of the uneven distribution of the phytoplasma in the vine. Although there is no information on phytoplasma distribution in a grapevine with AGY symptoms, our experience is that samples must be taken from symptomatic areas of the vine, and even then not all samples are phytoplasma positive.

This problem with detection changing with time adversely affects epidemiology studies where it is necessary to sample for phytoplasmas throughout a season so that comparisons can be made between symptom development and the association with phytoplasmas. It is not known whether the increased ability to detect phytoplasmas in January and February reflects the normal increase in phytoplasma titre associated with a previous season's infection. Another explanation is that it may reflect an increase in titre resulting from a recent inoculation event. Alternatively, the phytoplasma titre may be constant at these times and the increase in detection is due to an increasing efficiency of the PCR test perhaps resulting from a reduction in host inhibitors or an increase in the rate of circulation of the phytoplasma in the phloem.

At the optimum sampling time in February 1996, there was no clear benefit afforded by the nested PCR. To understand this process we need to increase our understanding of phytoplasma titre and distribution in the host throughout the growing season. We also need to determine whether DNA extracts do inhibit the PCR more at some times than at other times. Although there is no clear benefit to using nested PCR, we found on occasion, that the nested PCR optimised our chances of detecting phytoplasma after the optimum sampling time had passed.

Grapevines with a range of symptoms which had been shown to be phytoplasma positive using the nested universal PCR test, were subjected to further PCR testing using the AGY phytoplasma specific test. Interestingly, not all samples were positive using this AGY phytoplasma specific test. The TBB phytoplasma control was not amplified and vines with symptoms of LSLC, even though some of these had symptoms of AGY, were negative. These results indicated that vines with LSLC and known to be phytoplasma positive, were not infected with the AGY phytoplasma. In contrast, BONFIGLIOLI *et al.* (1995) reported an association between LSLC (late AGY) and the AGY phytoplasma. The vines with LSLC had symptoms over the entire vine but AGY symptoms were localised on a few spurs only. Efforts were made to take samples from both the AGY affected area and from the rest of the vine. It is possible that the titre of the AGY phytoplasma was very low in the AGY symptomatic sample, and was below the detectable level in the AGY phytoplasma specific tests. One plant with symptoms of LSLC and AGY was positive in both the universal and AGY specific tests and one plant with symptoms of RG which was positive in the nested universal PCR test, was negative in the AGY phytoplasma specific test.

A number of approaches were used to identify the phytoplasmas, known as GP-A, amplified in vines with LSLC symptoms. One approach was to use different primer com-

binations and include a range of reference phytoplasmas, TBB, AGY, VK, and BN. As expected, the universal nested PCR tests amplified all phytoplasmas. The stolbur specific primers amplified AGY, VK and BN but not TBB or GP-A, indicating that GP-A was not a stolbur phytoplasma. The AGY phytoplasma specific primers amplified AGY phytoplasma only, but not the other phytoplasmas. These results reaffirmed that GP-A was different from the AGY phytoplasma.

RFLP analysis of GP-A with a wide range of known phytoplasmas used as references, showed that this phytoplasma was indistinguishable from the sunnhemp phyllody phytoplasma from Thailand. This phytoplasma belongs to the faba bean phyllody phytoplasma group (SCHNEIDER *et al.* 1995) which includes the TBB phytoplasma (GIBB *et al.* 1996). CONSTABLE *et al.* (1998) also reported detection of a tomato big bud (TBB) type phytoplasma in grapevine, often in association with LSLC symptoms and occasionally with AGY symptoms, however there was no strong association between symptoms of LSLC and phytoplasma. BONFIGLIOLI *et al.* (1995) reported an association between symptoms of LSLC (late AGY) and the AGY phytoplasma but there was no report of the TBB phytoplasma in this study.

Grapevine samples showing AGY symptoms were collected from the Ovens Valley region in Victoria. Relatively small isolated pockets of Chardonnay are grown in this region which is elevated and experiences cooler temperatures than Chardonnay grown in the Sunraysia district. RFLP analysis showed that the phytoplasma associated with AGY symptoms is closely related to but distinguishable from the AGY phytoplasma, nor is it similar to any of the other reference phytoplasmas used. PADOVAN *et al.* (1996) compared the AGY phytoplasma from Chardonnay and Riesling vines collected from different regions in two Australian states but found no variation by RFLP analysis. This is the first report of an "AGY variant" phytoplasma and sequence analysis will be required for further characterisation.

Although other workers have demonstrated a clear association between the AGY phytoplasma and symptoms of AGY (MAGAREY and WACHTEL 1986; BONFIGLIOLI *et al.* 1995; PADOVAN *et al.* 1995, 1996; DAVIS *et al.* 1997; CONSTABLE *et al.* 1998), there have been few studies that have tested large numbers of grapevines with a range of symptoms and combinations of symptoms, especially LSLC and RG. In this study, almost 700 symptomatic vines were tested for phytoplasma, along with 381 asymptomatic vines. Interestingly, 3 % of asymptomatic vines were phytoplasma positive, with the majority of those being the AGY phytoplasma and one the TBB type phytoplasma. There may be a number of explanations for this result. Vines with detectable levels of phytoplasma can be asymptomatic or resistant or alternatively these vines may have gone on to show symptoms, *i.e.* in some cases phytoplasma may be detectable by the diagnostic test before symptoms appear. Symptoms of AGY do not generally involve the whole vine so it is also possible that a small symptomatic spur was removed and leaves near that area sampled for testing may not have shown clear symptoms. This is an unlikely explanation for vines with symptoms of LSLC or RG where the whole vine is usually affected.

Vines showing typical AGY symptoms showed a strong association with phytoplasma, the vast majority of which was the AGY phytoplasma. Some of the vines with symptoms of AGY were associated with the AGY "variant" phytoplasma but this was confined to vines from the Ovens Valley region. Occasionally vines with symptoms of AGY had the TBB type phytoplasma. It is possible that these vines had levels of the AGY phytoplasma that were too low to be detected. It is not known whether the TBB phytoplasma can also cause AGY symptoms because this phytoplasma is found so infrequently in vines with AGY symptoms, the great majority of which have the AGY phytoplasma. It is possible the TBB type phytoplasma is not associated with any particular disease and may just be asymptomatic in the vine.

Vines with both AGY and RG symptoms also showed a strong association with the AGY phytoplasma and again, the TBB type phytoplasma was detected infrequently in these vines. A small number of vines with RG symptoms (5 %) were phytoplasma positive and all of these were the AGY phytoplasma. It is possible that these vines had symptoms of AGY which were overlooked. Taking into account the background 3 % of asymptomatic vines shown to be phytoplasma positive, there is no strong evidence that RG symptoms are caused by phytoplasmas. Only 4 % of vines with both RSG and LSLC symptoms were phytoplasma positive and the phytoplasma was TBB. In other studies, RG symptoms have been associated with climatic and cultural factors (WILSON 1995, 1997; WILSON and HAYES 1996) and although vines with RG symptoms have been tested for phytoplasmas, there is also no strong evidence to suggest that this disease is associated with phytoplasmas (BONFIGLIOLI *et al.* 1995; PADOVAN *et al.* 1995; CONSTABLE *et al.* 1998).

Few vines with LSLC symptoms only were phytoplasma positive (10 %) however, of these, there appeared to be more vines with the TBB phytoplasma than with the AGY phytoplasma. It is possible that some of these vines had localised AGY symptoms which were not observed during sampling, or were removed during harvest. This would account for the occasional AGY phytoplasma but this amounted to 3 % which is not strong evidence to support the view that the AGY phytoplasma causes LSLC symptoms. This is in contrast to the report by BONFIGLIOLI *et al.* (1995) in which 81 % of vines with LSLC symptoms, refererred to as 'late AGY', had detectable levels of the AGY phytoplasma. In this study the phytoplasma was assumed to be the AGY phytoplasma but it may have been the TBB phytoplasma. Even so, 81 % is much higher than our 10 % and on the basis of our results we conclude that there is no strong evidence to suggest that LSLC symptoms are caused by phytoplasma. This is supported by the low incidence of phytoplasma in vines with both RG and LSLC symptoms.

There is no clear understanding of the relationship between the TBB type phytoplasma and diseases in grapevine (CONSTABLE *et al.* 1998). In this study, 26 % of vines with symptoms of both AGY and LSLC were phytoplasma positive, half were TBB and half were the AGY phytoplasma.

Of the 11 vines with symptoms of all three disorders, two had TBB and three had the AGY phytoplasma. There is, however, no overwhelming evidence to suggest that the TBB phytoplasma is associated with any particular disease in grapevine and it is quite possible that it is present at low levels in grapevine but does not necessarily cause disease. It would be important however, to monitor a cohort of vines over a number of seasons to determine whether, in fact, vines with the TBB phytoplasma, eventually do show symptoms of a disease. It is possible that infection with the TBB phytoplasma does not cause disease for some time and so any screening in one season will not necessarily reveal a strong correlation between phytoplasma and disease.

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