Natural Spread and Molecular Analysis of Grapevine Leafroll-Associated Virus 3 in Australia

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ABSTRACT

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Natural spread of grapevine leafroll disease was observed in a Pinot Noir clonal evaluation trial in South Australia. The trial consisted of 13 clones with the spread apparently initiated from 3 leafroll-infected clones: Antav 543, Geisenheim 20, and Bourgogne H199A. The occurrence of grapevine leafroll-associated virus 3 (GLRaV-3) was suspected. All 104 vines in the trial were tested by enzyme-linked immunosorbent assay using antibody to GLRaV-3 and by slot blot hybridization analysis using double-stranded RNA as target and labeled GLRaV-3–specific cDNA as probe. Both tests linked GLRaV-3 with the disease spread and detected the infection prior to the onset of symptoms. A cDNA clone from an Italian isolate of GLRaV-3 hybridized in Northern blots with three major dsRNAs of 19.5, 1.9, and 0.9 kbp extracted from leafroll-infected vines. The cloned cDNA insert of approximately 1 kbp was sequenced, and a set of primers designed based on the sequence was used to obtain a corresponding polymerase chain reaction product from the Antav 543 isolate grown in Australia. The nucleotide sequence of the cDNA clones from the two isolates of GLRaV-3 showed 99.5% identity and contained an open reading frame (ORF) encoding a putative protein with a molecular mass of 20.4 kDa with no significant homology to known protein sequences. This ORF was mapped near the 3′-end of the plus strand viral genome and had a 3′-untranslated AU-rich region of approximately 347 nucleotide residues.

Additional keyword: virus indexing.

Leafroll is an economically important graft-transmissible disease of grapevines and causes yield losses of up to 40% (18). The disease etiology is not well established, but filamentous closterovirus-type particles commonly are associated with the phloem tissue of infected vines (6,8,10,19). Extensive serological studies of leafroll disease have shown an association of six types of viruses, all of which resemble closteroviruses, with the disease (2,6,8,19). Grapevine leafroll-associated virus 3 (GLRaV-3) is the only type reported to spread in the field, and mealybugs have been reported as natural vectors (4,5,12,17). Here, we provide further evidence linking grapevine leafroll spread to GLRaV-3 using two independent techniques, and we characterize the 3′-region of the viral genome.

MATERIALS AND METHODS

Source of plant material. The vines were from a clonal evaluation trial of 13 Pinot Noir clones that had been subjected to heat therapy and planted at the Nuriootpa Research and Advisory Center, Nuriootpa, South Australia, in 1986. The clones were randomized with eight replicates, and the plot was surrounded by symptomless buffer vines. Table 1 lists the 13 clones used and their countries of origin. Duplicate samples from each vine were collected during autumn in two consecutive years. Samples of a healthy Emperor seedling and a Sultana (Thompson seedless) H5 clone infected with a mild leafroll (18) were from vines grown in a glasshouse in Adelaide, South Australia. Samples from other grapevine cultivars were collected from Nuriootpa or from a quarantine glasshouse at the CSIRO Laboratory in Merbein, Victoria, Australia.

Biological assay. A graft inoculation assay for leafroll and a range of other virus-like agents infecting grapevine was carried out according to Hewitt et al. (7) using the following indicators: Cabernet Franc, Mission seedling, and Baco 22A for leafroll; Mataro and Mission seedlings for yellow speckle; Baco 22A for flavescence dorée; *Vitis rupestris* 'St. George' for fanleaf; and *V. riparia* 'Gloire' for vein mosaic. Two successful indicator/candidate graft combinations and one healthy control were used for each assay. Symptoms were recorded over a minimum of three consecutive seasons.

Serological assay. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed essentially as described by Clark and Adams (3). A monoclonal antibody and its conjugate specific for GLRaV-3 were obtained from Bioreba AG, Basel, Switzerland. Each vine was tested at least twice by extracting petioles and midribs of mature leaves. Tissue extracts were prepared according to the instructions provided with the kit, except that the tissue was ground in liquid nitrogen prior to the addition of extraction buffer. Absorbance was recorded at 405 nm using an automatic microplate reader (Bio-Rad Laboratories, Rich-

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mond, CA). Samples with absorbance readings over three times that of the healthy control were considered positive.

Double-stranded RNA extraction. Extraction of double-stranded RNA (dsRNA) from leafroll-infected grapevine tissue for Northern blot hybridization was carried out as described (11). For slot blot hybridization, 0.15 g of midrib tissues from mature leaves was placed in a small plastic bag, mixed with 0.5 ml of extraction buffer (11), sealed, and smashed with a hammer on a steel plate. Binding of the dsRNA to CF-11 cellulose powder, washing, and elution were carried out in an Eppendorf tube. The dsRNA was eluted in 0.6 ml of STE buffer (50 mM Tris-HCl, pH 7.0; 100 mM NaCl; 1 mM EDTA). NaOH was added to a concentration of 50 mM, and the extract was filtered through Zeta-probe nylon membrane (Bio-Rad) using a vacuum manifold. The RNA was cross-linked to the membrane by exposure to UV light for 1 min.

Nucleic acid hybridization. Northern blot analysis was carried out after electrophoresis of dsRNA samples in 6% polyacrylamide gels in Tris-acetate-EDTA buffer under nondenaturing conditions (9). The dsRNA was electroblotted onto Zeta-probe GT nylon membranes (Bio-Rad) according to the manufacturer's protocol. The membranes were soaked in 50 mM NaOH and 10 mM NaCl for 5 min, rinsed twice in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) for 5 min, and cross-linked with UV light. Nucleic acid hybridization was carried out using a GLRaV-3–specific cDNA clone obtained from Italy (14), referred to as 23ds, with an insert size of approximately 1 kbp. For probe synthesis, the insert was excised from pGEM3Zf+ using restriction endonucleases *Eco*RI and *Hin*dIII (15) and was labeled with ^{32}P using a random hexamer kit (Bresatec, Adelaide, South Australia) according to the manufacturer's instructions. Prehybridization, hybridization, and washing of the membranes were carried out at 65°C according to the instructions provided by Bio-Rad. The blots were exposed overnight to a Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) at –70°C using an intensifying screen.

DNA sequencing, reverse transcription, and polymerase chain reaction. Dideoxynucleotide chain termination sequencing (16) was used to sequence the ends of a GLRaV-3 DNA insert in pBluescript II SK+ plasmid (Stratagene, La Jolla, CA) using a M13 universal primer (–40) or T3 primer (United States Biochemical Corporation, Cleveland). Two specific primers, S1 and S2 (Table 2), were synthesized from the sequence information and used to complete the sequence of the 23ds clone.

The reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out in two steps. In the first step, samples of dsRNA (50 to 100 ng) from the Australian isolate were mixed with 1 µg of P1 primer (Table 2) in 5 µl of water, sealed in a capillary tube, and placed in a boiling water bath for 5 min. The tube was allowed to cool to 42°C over a period of 30 min and was subjected to a reverse-transcription reaction in a total volume of 20 µl using AMV reverse transcriptase, according to the instructions provided by the supplier (Riboclone kit, Promega, Madison, WI), except that KCl was excluded. In the second step, a PCR reaction (13) was performed in a reaction volume of 20 µl containing 6 µl of the above cDNA reaction mix, 200 µM of each of the four nucleotides, 1.5 mM $MgCl_2$ and 1 µM of each of the P1 and P2 primers (Table 2). The forward primer (P1) contained an extra six nucleotide residues added to its 5′-end to provide a restriction site for *Xba*I for subcloning, and the reverse primer (P2) contained an extra eight nucleotides at its 5′-end to provide a restriction site for *Eco*RI. PCR tubes were subjected to an initial denaturation temperature of 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min.

The size of the 3′-end region of GLRaV-3 RNA was estimated by primer extension on the dsRNA template in a reverse-transcription reaction, as mentioned above, using the specific primer (3′ primer; Table 2).

RESULTS

Biological assay and spread of leafroll symptoms. The results of biological assays of 12 Pinot Noir clones using a number of indicator vines are summarized in Tables 1 and 3. Three of the clones—Bourgogne H199A, Geisenheim 20/NX, and Antav 543—induced typical leafroll symptoms on Cabernet Franc and Mission. Graft inoculation of the remaining clones did not induce symptoms in any of the indicators (Tables 1 and 3).

TABLE 1. Names and sources of Pinot Noir grapevine clones assessed

Clone[a]	Source	Accession no.
D5V12 (2051)	Australian Industry standard clone, originally from the University of California, Davis	IS 642051
Bourgogne H80A	Introduced into Australia in the mid-1970s, from the Lausanne Institute, Switzerland, originally from Burgundy	AS 860331
Bourgogne H140A		AS 860300
Bourgogne H199A*		…[b]
Oberlin H120A	Introduced from the Lausanne Institute in the mid-1970s, originally from north Germany	AN 720301
Oberlin H120B		AN 720302
Antav 151, H120B	Introduced in the mid-1970s from ANTAV, France	IN 700187
Antav 167, H120B		IN 700188
Antav 542		IN 700185
Antav 543*		IN 700186
20/NX/Geisenheim*	Introduced from Geisenheim, Germany	IN 680199
Cortailloid H120B	Introduced from the Lausanne Institute, originally from France	AS 860310
MV6 H170A	Australian clone, from the Hunter Valley	AS 742340

[a] * indicates the clone was infected with leafroll as shown by biological indexing (Table 3).
[b] No accession number was given.

TABLE 2. Grapevine leafroll-associated virus 3-specific oligonucleotide primers used

Primer[a]	Size (nt)	Nucleotide position[b]	Sequence[c]
P1	36	38–68	5'-gctctaGACCACACTGAATATTTT GCACGATAAGGC-3'
P2	34	1001–1026	5'-cggaattcGCCGCCTAGGTCCAAAC TTTAATTGG-3'
S1	21	356–376	5'-TCTACGATAGATGCTTTCGCG-3'
S2	18	692–709	5'-CCGATTAACGTTACCTCG-3'
3′	20	900–919	5'-GACTAAACTCTACCTCACGG-3'

[a] P1, S1, and 3′ are sense primers, and P2 and S2 are antisense primers.
[b] Nucleotide position in Figure 3.
[c] Lowercase letters indicate the extra nucleotide residues added for cloning polymerase chain reaction fragments.

TABLE 3. Biological assay of 12 Pinot Noir grapevine clones for leafroll and other graft-transmissible disease agents[a]

Pinot Noir clone[b]	Indicator vines[c]					
	Vitis riparia 'Gloire'	Mataro	Cabernet Franc	Mission seedling	BACO 22 A	*Vitis rupestris* 'St. George'
Bourgogne/199A	0	0	2	2	0	0
20/NX/Geisenheim	0	0	2	2	0	0
Antav 543	0	0	2	2	0	0

[a] Results of 3 years of observation using two inoculated vines per assay.
[b] Grapevine clone D5V12 was not included in indexing. The remaining nine clones of those listed in Table 1 were indexed negative.
[c] 0 and 2 indicate the number of symptomatic vines.

The positions of the infected vines in the randomized trial at the time of planting in 1986 are shown in Figure 1A. A sporadic spread of symptoms was first observed in 1991 and continued in subsequent years. Figure 1 shows individual vines inspected for symptoms in 1986, 1993, and 1994. The pattern of disease spread was nonrandom; newly infected vines formed a cluster with the previously infected plants within a row. Symptoms appeared in Antav 543 vines about 4 weeks earlier than in the other leafroll-infected clones, and the grape yield was about 50% of the other leafroll-infected clones (data not shown). This yield difference may be due to coinfection of Antav 543 with GLRaV-3 (discussed below) and other viruses as indicated by the presence of dsRNA bands in addition to those hybridizing with the GLRaV-3 probe in Northern blots (data not shown).

Association of GLRaV-3 with leafroll spread. To identify the type of leafroll virus associated with disease spread, a number of symptomatic vines from replicates 7 and 8 of the trial (Fig. 1) and a healthy Emperor seedling were examined by slot blotting the dsRNA extracts onto a nylon membrane and hybridizing with a GLRaV-3–specific cDNA probe. RNA extracts from the originally infected vines (Fig. 1A: vine 4, replicate 7 [V4R7], V7R7, V5R8, and V8R8) as well as the newly infected vines (Fig. 1B: V5R7, V6R7, V9R8, and V10R8) reacted with the probe (Fig. 2A). The probe did not hybridize to an extract from a healthy Emperor seedling. To evaluate the possibility of detecting disease spread prior to the onset of symptoms, the vines in the entire trial were tested by slot blot hybridization analysis and by ELISA. The techniques detected infection in all symptomatic vines as well as in 11 symptomless vines that all developed symptoms in the subsequent season (Fig. 1C; Table 4). The results obtained by both techniques were in agreement, except for the symptomless vine, V9R5, which gave a positive reaction using slot blot analysis but a negative reaction using ELISA (Table 4). The results of this epidemiological study associate GLRaV-3 with spread of the disease symptoms observed and provide circumstantial evidence for the role of GLRaV-3 in the etiology of leafroll disease.

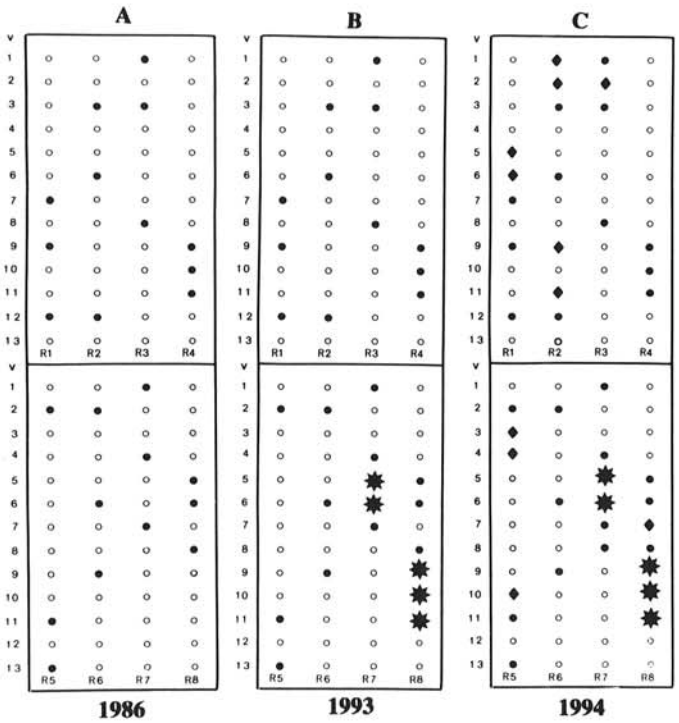

Fig. 1. Symptom spread of leafroll disease in a Pinot Noir clonal trial at Nuriootpa, South Australia. Thirteen clones were planted in eight replicates (R1 to R8) in four rows. **A,** Closed circles indicate infected grapevines in 1986 (planting time). New infections are shown **B,** with an asterisk in 1993 and **C,** with diamonds in 1994. Open circles show vines without symptoms.

Northern blot analysis of dsRNA from the infected vines from Australia and Italy, using the GLRaV-3 probe, revealed three major dsRNA species of 19.5, 1.9, and 0.9 kbp (Fig. 2B). A similar dsRNA pattern was obtained when the extracts from the following grapevine cultivars were examined: Italia, Keknyelu, Roupeiro Cachudo, Malvasia di Candia, Tinta Carvalha, and Cabernet Franc clone 1329 (results not shown). No hybridization signals were observed in Northern blots when dsRNA extracts were used from a leafroll-infected Sultana H5 vine (18), a Cabernet Franc vine graft-inoculated with the RdL isolate of leafroll (11), a Sultana H23 vine, a Manik Chaman vine, or a healthy Emperor vine. These leafroll-infected vines (11,18) are likely to carry other GLRaV types (N. Habili, *unpublished data*).

To compare the relationship between the Australian, Antav 543, and Italian isolates of GLRaV-3 at the nucleotide sequence level, the cDNA clone 23ds of the Italian isolate was sequenced (Fig. 3). This allowed us to obtain a set of primers for use in a RT-PCR reaction with the Australian dsRNA template. A PCR product of 988 nucleotides from the Australian isolate of GLRaV-3 was cloned and sequenced. The nucleotide sequence of the two isolates showed 99.5% identity in this part of the genome with five nucleotide substitutions (Fig. 3). An open reading frame (ORF) encoding 183 amino acid residues was present in this region. None of the nucleotide substitutions caused any change in the sequence of the putative translation product. This ORF has the

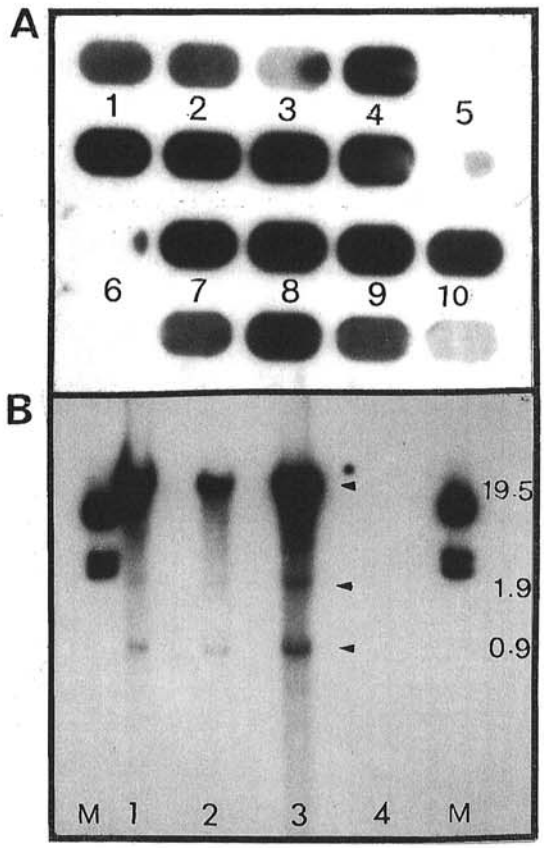

Fig. 2. Hybridization of double-stranded RNA (dsRNA) extracted from selected grapevines with a ^{32}P-labeled grapevine leafroll-associated virus 3 (GLRaV-3)-specific probe. **A,** Slot blot hybridization analysis. Samples 1 to 4, dsRNA extracted in duplicate from infected vines 4 and 7 in replicate 7 and 5 and 8 in replicate 8; samples 5 and 6, healthy Emperor seedling and Sultana clone H5, respectively, used as negative controls; samples 7 to 10 from vines 8 to 11 in replicate 8 of Figure 1. **B,** Northern blot analysis. Lane 1, dsRNA extracted from vine 6 of replicate 7 (Fig. 1); lane 2, dsRNA extracted from vine 11 of replicate 8; lane 3, a dsRNA extract from GLRaV-3–infected tissue from Italy as a positive control; lane 4, dsRNA from Sultana H5 as a negative control; and lane M, ^{32}P-labeled λ DNA digested with the endonuclease *Hin*dIII was used as a molecular size marker. The size of major dsRNA bands is shown in kilobase pairs.

potential to encode a protein with a molecular mass of 20.4 kDa with no significant homology to any known protein sequences.

The ORF is followed by a noncoding AU-rich region (68% AU, Fig. 3). Primer extension analysis, using a primer (3′ primer, Table 2) and dsRNA templates isolated from the infected vines, mapped the ORF close to the 3′ end of the plus strand viral RNA (Fig. 4). The size of the 3′ noncoding region was estimated to be 347 nucleotide residues, extending the sequence in Figure 3 by approximately 18 residues (Fig. 4).

DISCUSSION

We observed natural spread of grapevine leafroll in Pinot Noir vines in Australia. GLRaV-3 was detected in infected vines and was correlated with the spread using ELISA and nucleic acid hybridization techniques (Fig. 2; Table 4). Both techniques detected the disease in vines before the natural onset of symptoms. This early detection offers a valuable practical alternative to biological indexing. The techniques are especially useful in white

TABLE 4. Detection of grapevine leafroll-associated virus 3 in individual Pinot Noir grapevines by symptom expression, nucleic acid hybridization, and enzyme-linked immunosorbent assay (ELISA)

	R1			R2			R3			R4			R5			R6			R7			R8		
Vine[a]	s[b]	d	e	s	d	e	s	d	e	s	d	e	s	d	e	s	d	e	s	d	e	s	d	e
V1	−	−	−	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
V2	−	−	−	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−
V3	−	−	−	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−
V4	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−
V5	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
V6	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
V7	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
V8	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+
V9	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	−	−	−	+	+	+
V10	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+
V11	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+
V12	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
V13	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−

[a] Positions of vines (V) and replicates (R) are as in Figure 1.

[b] Symptoms (s) were recorded in 1995, and nucleic acid hybridizations (d) and ELISA (e) were carried out in 1994. + indicates a positive reaction; − indicates no reaction.

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1     AGCUUGCAUG CCUGCAGGUC GACUCUAGAG GAUCCCCCGA CCACACUGAA UAUUUUGCAC
61    GAUAAGGCUU UCACGAGGAA AAACCUGAGU AACAAAGAUA AGGGGUCCUC GGAUUUAAGG
                                                 G
121   AUAGAAGAAA AUUUUUUAAA AUCCGCCGUA GAUACAGAUA CGAUUUUGAA UGGAUAAAUA
181   UAUUUAUGUA ACGGGGAUAU UAAACCCUAA CGAGGCUAGA GACGAGGUAU UCUCGGUAGU
241   GAAUAAGGGA UAUAUUGGAC CGGGAGGGCG CUCCUUUUCG AAUCGCGGUA GUAAGUACAC
                                          U
301   CGUUGUCUGG GAAAACUCUG CUGCGAGGAU UAGUGGAUUC ACGUCGACUU CGCAAUCUAC
                                          U
361   GAUAGAUGCU UUCGCGUAUU UCUUGUUGAA AGGCGGAUUG ACUACCACGC UCUCUAACCC
421   AAUAAACUGU GAGAAUUGGG UCAGGUCAUC UAAGGAUUUA AGCGCGUUUU UCAGGACCCU
481   AAUUAAAGGU AAGAUUUAUG CAUCGCGUUC UGUGGACAGC AAUCUUCCAA AGAAAGACAG
541   GGAUGACAUC AUGGAAGCGA GUCGACGACU AUCGCCAUCG GACGCCGCCU UUUGCAGAGC
601   AGUGUCGGUC CAGGUAGGGA AGUAUGUGGA CGUAACGCAG AAUUUAGAAA GUACGAUCGU
            U
661   GCCGUUAAAA GUUAUUGAAA UAAAGAAAAG ACGAGGUAAC GUUAAUCGGU AUGUUUAUUA
721   GUUACUAUUU AUAUGCUCUA AUUAGCAUAU GUAGAAAAGG GGAAGGUCUG GGAACUAUUA
781   AUAGGUAAAG ACUCUUCAAU AAAUUUGAAA UAAACAAAAG UAAGAAAAAU GAAAUAAUUA
841   GGCUAGUCUU UUUGUUCGUU UUUCGUUUUU GUAGAAUAGG UUAUAUUUCG AGGUAAGAUG
                                                                   C
901   ACUAAACUCU ACCUCACGGU UUAAUACUCU GAUAUUUGUA AAAUUAGUCC GUAAAGUCGA
961   UAGUGAUAUU AUAUUAGUAU AGUAUAAUAA ACGCCAAAAU CCAAUUAAAG UUUGGACCUA
1021  GGCGGCUCUU ACGAGGCUAA CUUAUCG
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Fig. 3. Nucleotide sequence of the plus strand of the Italian isolate of grapevine leafroll-associated virus 3 (GLRaV-3) obtained from the 23ds cDNA clone. Boxed letters indicate putative initiation and termination codons spanning the open reading frame (indicated by double line). The positions of primers (P1 and P2, Table 2) used in reverse-transcriptase polymerase chain reaction are underlined. Nucleotide differences between the 3′ regions of the Australian and the Italian isolates of GLRaV-3 are indicated.

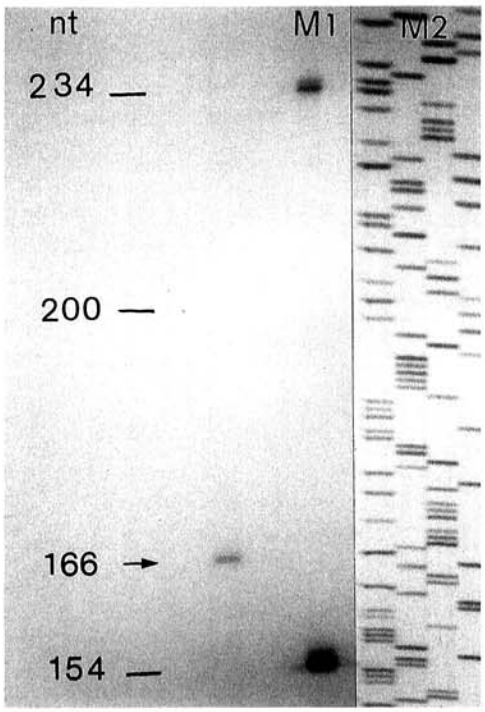

Fig. 4. Primer extension of the 3′-end region of the positive strand of double-stranded RNA extracted from grapevines infected with the Australian isolate of grapevine leafroll-associated virus 3 (GLRaV-3). The DNA product of 166 nucleotides (arrow) was synthesized using the 3′ primer (Table 2) and analyzed in a sequencing gel using DNA size markers (M1) (DNA VI, Boehringer GmbH, Mannheim, Germany) and a DNA sample from a dideoxynucleotide sequencing reaction mixture (M2) as a size ladder.

vine cultivars in which leafroll symptoms are difficult to assess visually.

A similar pattern of spread involving GLRaV-3 has been observed in a Pinot Noir clone, D5V12a, at Irymple, Victoria, Australia (G. Fletcher, *personal communication*, and A. Ewart, *unpublished data*). The spread of leafroll among Pinot Noir vines in South Australia and Victoria was first observed in 1991, at least 5 years after planting in the field. The mode of disease transmission remains unknown. Mealybugs have transmitted GLRaV-3 in Sicily (12), Israel (17), South Africa (4), and the United States (5). However, these insects could not be found on the vines in our experimental plot. Mealybugs also were absent in a vineyard in northern Italy where the spread of GLRaV-3 has been linked to the occurrence of the scale insect *Pulvinaria vitis* (1). The results presented here indicate that the Australian and Italian isolates of GLRaV-3 are identical in their dsRNA patterns (Fig. 2B), and they show a high degree of homology in 1 kb of their cDNA nucleotide sequence that encompasses an overlapping putative gene at the 3′ end of the genome (Fig. 3).

The pattern of leafroll spread observed here frequently appears to involve neighboring vines within rows in the plot (Fig. 1), implicating the involvement of a slow-moving vector. The role of soilborne vectors, mites, and pollen in the transmission of the disease cannot be ruled out nor can transmission by root grafting.

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