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Short communication

Identification of divergent variants of *Grapevine virus A*

D.E. Goszczynski and A.E.C. Jooste
Plant Protection Research Institute, Agricultural Research Council, Private Bag X134, Pretoria 0001, South Africa (Fax: +27128088299; E-mail: nipbdg@plant1.agric.za)

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Abstract

Eight isolates of *Grapevine virus A* (GVA), which induced different symptoms in leaves of *Nicotiana benthamiana*, were recovered from various grapevines. The dsRNA patterns of two isolates, which consistently induced mild vein clearing (referred here as mild isolates of GVA) were similar, but different from those of other isolates of GVA. Analysis based on overall nucleotide (nt) sequence identity in the 3′ terminal part of the GVA genome, comprising part of ORF3 (putative movement protein, MP), entire ORF4 (capsid protein, CP), entire ORF5 and part of 3′UTR, revealed that GVA isolates separate into three groups (I, II, III), sharing 91.0–99.8% nt sequence identity within groups and 78.0–89.3% nt sequence identity between groups. Mild isolates of the virus were group III and shared only 78.0–79.6% nt sequence identity with the other isolates. The comparison of predicted amino acid sequences for MP and CP revealed many amino acid alterations, revealing distinct local net charges of these proteins for mild isolates of the virus. Based on both conserved and divergent nt regions in the CP and ORF5, oligonucleotide primers were designed for the simultaneous RT-PCR detection of all GVA isolates and for the specific detection of the most divergent virus variants represented here by mild isolates of the virus.

Grapevine virus A (GVA) is the type member of the *Vitivirus* genus (Martelli et al., 1997). The virus has filamentous particles about 800 nm long that contain positive sense single-stranded RNA, of 7349 nucleotides (nt), excluding a poly(A) tail at the 3′ terminus. The genome of GVA is organized into five open reading frames (ORF1–5) (Minafra et al., 1997), which encode putative replicase (ORF1), putative movement protein (MP) (ORF3), capsid protein (CP) (ORF4) and putative nucleic acid-binding protein (ORF5), respectively. The function of the polypeptide encoded by ORF2 is not known. Although GVA was reported more than 20 years ago (Conti et al., 1980), and is one of the most frequently detected viruses in vineyards worldwide, its involvement in grapevine diseases is still not clear. The results obtained in different laboratories suggest that the virus is implicated in the aetiology of Kober stem grooving (KSG) (Garau et al., 1994; Chevalier et al., 1995; Choueri et al., 1997), which is one of the four economically important grapevine diseases of the rugose wood complex (RW) (Minafra, 2000). The isolation of GVA and its transmission back to grapevines (fulfillment of Koch's postulates) is crucial for understanding the role of this virus in grapevine diseases. As strains of viruses with different pathogenicities to plants are common, and are sometimes present in a single plant (Moreno et al., 1993; Ayllon et al., 2001), detailed biological and molecular characterization of GVA is needed. The advantage of the study of GVA is that the virus can be transmitted from grapevines to herbaceous plants by insect vectors (Boscia et al., 1997), or mechanically (Monette et al., 1990; Goszczynski et al., 1996). Recently, single-strand conformation polymorphism (SSCP) analysis of GVA isolates recovered in *Nicotiana benthamiana*, revealed extensive molecular heterogeneity of the virus (Goszczynski and Jooste, 2002). In this paper, we present results of biological and molecular studies of these isolates, which lead to the identification of divergent molecular variants of GVA consistently

inducing mild symptoms in *N. benthamiana*. RT-PCR for specific detection of these variants was developed.

Mechanical transmission of GVA from various grapevines to *N. benthamiana* was carried out as described by Goszczynski et al. (1996). Preparations of viruses partially purified from leaf petioles or cane phloem from each grapevine were used for inoculation of eight *N. benthamiana* plants. GVA-infected single *N. benthamiana* plants obtained in this experiment are referred to here as virus isolates. GVA isolates GTR1-1, GTR1-2 and GTR1-3 were recovered from a grapevine cv. Shiraz (referred here as Shiraz GTR1), and those of GTG11-1, P163-1, JP98 and MSH18-1 from grapevines cvs Shiraz (GTG11), Cinsaut Blanc clone P163/12, Waltham Cross (JP98) and Shiraz (MSH18), respectively. Isolate 92/778, recovered from grapevine cv. Cabernet Sauvignon, was obtained earlier by Goszczynski et al. (1996). Each of the isolates was sub-inoculated to eight new plants of *N. benthamiana* every 20–30 days for a total of eight serial passages.

Immunosorbent electron microscopy (IEM) studies of *N. benthamiana* plants infected with GVA were performed as described by Goszczynski et al. (1996).

DsRNA was isolated from virus-infected *N. benthamiana* or grapevines (Hu et al., 1990). To increase purity of dsRNA, the preparations were passed through CF11 cellulose columns twice (Valverde et al., 1990). Two ml preparations of dsRNA in ethanol were centrifuged in a Sigma 101M microcentrifuge at 15,000 rpm for 20 min and the pellets were each resuspended in 10 µl sterile deionised water and stored at −80 °C until used. DsRNA was electrophoresed in 6%, 0.75 mm acrylamide/N,N′-methylene-bis-acrylamide (29.2/0.8) gels in TAE buffer (Valverde et al., 1990) at room temperature for 3.5 h, using Mini-protean II dual slab cell (Bio-Rad). The gels were stained with 0.5 µg ml^{-1} EtBr in TAE buffer, for 10 min, and photographed using a digital UVP system (Vacutec-Grab-It Version 2.5), and printed using a SONY Digital Graphic Printer UP-D890.

RT-PCR amplification of the 3′ terminal part of the genome of each isolate of GVA, cloning, sequencing and sequence analyses were done as described by Goszczynski and Jooste (2002). The oligonucleotide primer pair MP (5′-GCCAGAGGTGTTT GAGACAAT-3′), CPdt (5′-TTTTTGTCTTCGTGT GACAACCT-3′) was used (De Meyer et al., 2000).

Primers for the simultaneous RT-PCR detection of all molecular variants of GVA-primer pair GVA6591F (5′-GAGGTAGATATAGTAGGACC TA-3′) and GVA6862R (5′-TCGAACATAACCTG TGGCTC-3′) (PCR product 271 bp), and primers designed for the specific detection of the virus variants represented here by mild isolates – primer pair GVA6591F (see above) and GVA6906 (5′-CCTCCT GCAGAGTTAAGGTC-3′) (PCR product 315 bp), were designed manually using the obtained GVA sequence data. The conditions of RT-PCR for using these primers were as follows: five µl of the dsRNA preparations were denatured at 99 °C for 5 min, cooled on ice for 2 min, and then briefly centrifuged. One or 4 µl of denatured dsRNA was used in 10 µl of reverse transcription reaction, depending on whether dsRNA was isolated from virus-infected *N. benthamiana* or grapevines. Ten µl reverse transcription reaction was prepared from sterile deionised water (4.25 or 1.25 µl) plus 2 µl 5× M-MLV RT reaction buffer (Promega), 1 µl dNTPs (10 mM each), 1 µl of hexanucleotide mix (Roche, Cat. No. 1277081), 0.25 µl RNasin (30 U µl^{-1}) (Promega), 0.5 µl M-MLV reverse transcriptase) (200 U µl^{-1}) (Promega). Reverse transcription was performed at 42 °C for 1.5 h. One µl of RT was used in 10 µl PCR reaction containing 6.575 µl of water, 0.125 µl Taq DNA polymerase (5 U µl^{-1}) (Promega), 1 µl 10× buffer A, 0.6 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs and 0.25 µl each of forward and reverse primers. Thermal cycling parameters were as follow: 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min and a final elongation at 72 °C for 5 min.

PCR-generated products were analyzed by electrophoresis in 1% agarose gels containing 0.5 µg ml^{-1} EtBr, in TAE buffer. The gels were photographed as described for polyacrylamide gels. Negative images of gels are shown.

Eight isolates of GVA transmitted mechanically from various grapevines to *N. benthamiana* induced four kinds of symptoms on leaves: (1) mild vein clearing; (2) vein clearing plus interveinal chlorosis; (3) vein clearing, interveinal chlorosis plus strong curling of top leaves; and (4) extensive 'patchy' necrosis. IEM study of these plants did not reveal any virus particles other than GVA. PAGE analysis showed that dsRNA patterns of two GVA isolates consistently inducing mild vein clearing only (isolates GTR1-1 and P163-1) are similar, and differ from other isolates of the virus (Figure 1). Interestingly, the comparison of electrophoretic mobility of dsRNAs suggested that isolate GTR1-3 comprises isolates GTR1-1 and GTR1-2. All three isolates were recovered

from grapevine cv. Shiraz in a single transmission experiment.

The 3′ terminal part of each GVA isolate was RT-PCR amplified using primers MP and CPdt, cloned and sequenced. At least three clones were sequenced for each isolate. Results showed that sequences of mild isolates of GVA (GTR1-1 and P163-1) were one nt shorter than in other isolates of the virus. The sequences of the primers were excluded from analysis. The remaining 942 nt and 943 nt fragments comprised 100 nt of ORF3, entire ORF4 and ORF5 and 49–50 nt of 3′UTR. Analysis based on overall nt sequence similarity results (Table 1) revealed that GVA isolates cluster into three groups (I, II, III), sharing 91.0–99.8% of nt identity within groups and 78.0–89.3% of nt identity between groups. Group III contained only mild isolates of the virus, which shared 78.0–79.6% of nt sequence identity with other isolates. The nt sequences of representatives of each group reported here have been deposited in the GenBank/EMBL database with the accession numbers AF441234 (isolate 92/778), AF441235 (isolate JP98) and AF441236 (isolate P163-1).

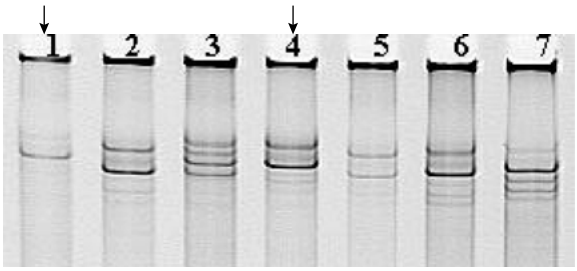

Figure 1. Patterns of dsRNA isolated from *N. benthamiana* infected with GVA isolates GTR1-1 (1), GTR1-2 (2), GTR1-3 (3), P163-1 (4), GTG11-1 (5), JP98 (6) and 92/778 (7). Arrows indicate dsRNA patterns of isolates GTR1-1 and P163-1, which induce mild symptoms on *N. benthamiana.*

Results of the analysis of the four clones of isolate GTR1-3 showed that nt sequences of three of them were almost identical to isolate GTR1-1 (99.8% of nt identity), and the sequence of one clone similar to isolate GTR1-2 (98.0% of nt identity). The two types of sequences found for isolate GTR1-3 are presented in Table 1 as GTR1-3A and GTR1-3B, respectively. This finding supported results of dsRNA analysis of isolate GTR1-3 (Figure 1), which suggested that the source plant contained two strains of GVA. The results agreed with those from the analysis of these isolates using a SSCP technique (Goszczynski and Jooste, 2002). Isolate GTR1-2 induced symptoms of vein clearing and interveinal chlorosis in leaves of *N. benthamiana*, which clearly differed from the mild vein clearing induced by isolate GTR1-1. That the isolates were recovered from a single grapevine cv. Shiraz (GTR1), suggests that this grapevine is infected with different biological variants of GVA.

Nucleotide divergences between isolates were randomly distributed within ORF5. However, clusters of nt sequence divergence, characteristic only for the mild isolates of GVA, were found in the C-terminal part of the MP (ORF3) and N-terminal half of the CP (ORF4) (not shown). Comparison of the predicted 198 aa sequence of the CP of mild and other isolates of GVA revealed four amino acid alterations, which determined a distinct local net charge of the CP for mild isolates (Figure 2B). At the positions of the neutral amino acids – threonine (T, position 30), asparagine (N, position 67) and serine (S, position 84), in the CP of mild isolates, charged amino acids – strong acidic

Table 1. Nucleotide (nt) identity of the 3′ terminal (942–943 nt) sequences of various GVA isolates

Group	Isolate	% Nucleotide identity[b]								
		GVA-MIN[a]	MSH18-1	92/778	GTG11-1	GTR1-2	GTR1-3B	JP98	GTR1-1	GTR1-3A
I	MSH18-1	92.8								
	92/778	92.0	93.7							
	GTG11-1	92.3	92.8	93.5						
II	GTR1-2	86.9	87.5	89.3	88.9					
	GTR1-3B	85.6	86.0	88.2	87.8	98.0				
	JP98	87.0	86.9	88.1	87.5	92.5	91.0			
III	GTR1-1	78.3	78.5	79.2	79.6	78.2	79.2	78.3		
	GTR1-3A	78.1	78.5	79.0	79.4	78.0	79.0	78.3	99.8	
	P163-1	78.2	79.1	79.4	79.3	78.3	79.3	78.2	97.7	97.7

[a]Isolate of GVA, of which the nt sequence was deposited in the GeneBank by Minafra et al. (1997), accession number X75433.
[b]Sequence data were analyzed using DNAMAN version 2.71 (Lynnon Biosoft, 1996, Quebec) software package.

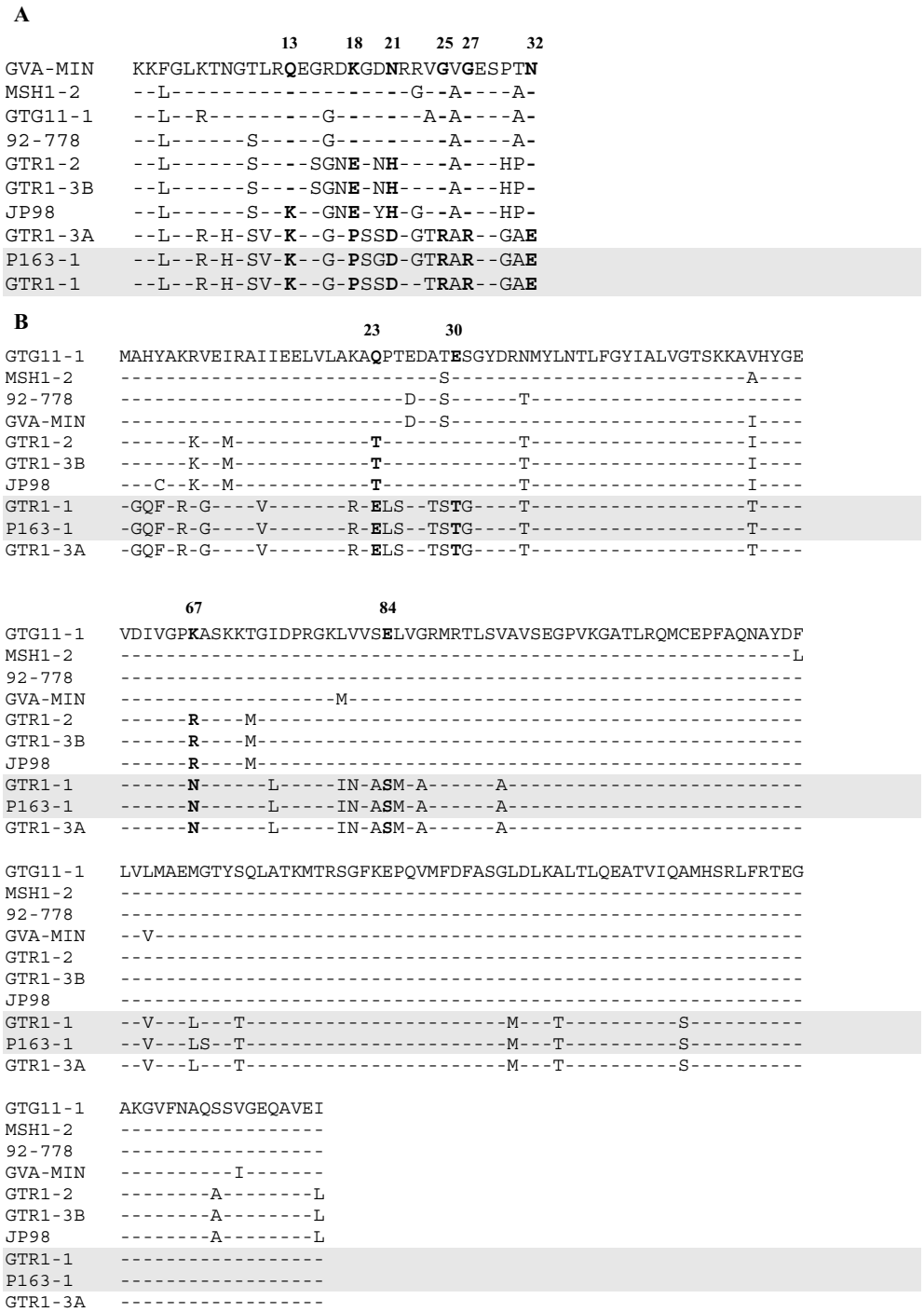

Figure 2. Comparison of predicted amino acid sequences of C-terminal part of MP (A) and entire CP (B) various isolates of GVA. Shadowed area shows predicted amino acid sequences of isolates P163-1 and GTR1-1, which induce mild symptoms on *N. benthamiana*.

glutamic acid (E), strong basic lysine (K) or arginine (R), were found in the CP of the other isolates of the virus. Only in one case, charged amino acid – strongly acidic glutamic acid (E, position 23), in the CP of mild isolates was substituted with neutral amino acids – glutamine (Q) or threonine (T), in the CP of the other isolates of the virus.

Although we cloned and sequenced only 100 nt of ORF3 (MP) and 49–50 nt of 3′UTR, which constitute about 12% and 72%, respectively, of the entire GVA, the results of sequence identity analysis revealed great divergence of nt sequences in these regions between mild and other isolates of the virus (65.0–73.0% and 49.0–51.0% of identity, respectively). The predicted 32 aa sequence for the fragment of ORF3 encoding the C-terminal part of MP revealed six alterations of amino acids (Figure 2A), which, analogous to CP, determined a distinct local net charge of this protein for mild isolates of the virus. Unlike in CP, however, at positions of charged amino acids – strongly basic lysine (K, position 13), strongly acidic aspartic acid (D, position 21) and glutamic acid (E, position 32), and strongly basic arginine (R, positions 25 and 27), in the MP of mild GVA isolates, neutral amino acids – glutamine (Q), asparagine (N) and glycine (G) or weakly basic histidine (H), in the MP of other isolates of the virus were found. In only one case, neutral amino acid – proline (P, position 18), in the MP of mild isolates was substituted with charged amino acids – strongly basic lysine (K) or strongly acidic glutamic acid (E), in the MP of the other isolates.

Although the meaning of the identified divergences in MP, CP and 3′UTR between GVA isolates is not known at this stage, they probably have physiological implications. The importance of MP, CP and 3′UTR for viral infectivity and pathogenicity is well documented for some plant viruses (Banerjee et al., 1995; Moreno et al., 1997; Bol, 1999). The mild symptoms induced in *N. benthamiana* by the most divergent variants (group III) of GVA is intriguing. However, since even a single nt substitution can change the pathogenicity of a virus (Banerjee et al., 1995), further study is required to answer the question of whether the mild reaction of *N. benthamiana* can be a biological indicator for this group of GVA variants.

Analysis of the variability of the 942–943 nt 3′ terminal fragment of the GVA genome revealed five longer (20 nt and more) regions in which nt sequences were highly conserved among all GVA isolates studied. The identified regions are between nt positions 6534–6562, 6591–6612, 6840–6859, 7006–7031 and 7072–7094. The first three are located in ORF3 (CP) and the last two in ORF5 of the virus. The sense and antisense oligonucleotide primers complementary to these regions were used in RT-PCR in different combinations. The results of RT-PCR using primer pair GVA6591F and GVA6862R, which consistently produced strong PCR products with expected molecular weight, for all GVA isolates are presented in Figure 3A. These new primers increased the pool of GVA-specific primers, which should be tested in different laboratories worldwide to confirm reliable detection of this virus.

High nt sequence divergence in CPs between group III and other groups of GVA isolates allowed us to develop RT-PCR for the specific detection of molecular variants of group III, represented in this study by mild isolates of GVA. Among seven GVA isolates from *N. benthamiana* tested by RT-PCR using primers GVA6591F and GVA6906R, strong DNA products, with expected molecular weight, were obtained only

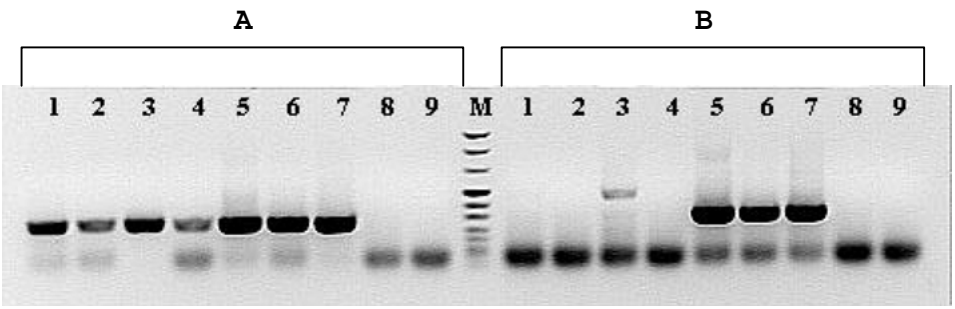

Figure 3. RT-PCR detection of GVA from *N. benthamiana* using oligonucleotide primers for simultaneous detection of all GVA isolates (A) and for the specific detection of the virus variants of group III represented in this study by mild isolates of the virus (B). The GVA isolates MSH18-1 (1), GTG11-1 (2), JP98 (3), GTR1-2 (4), P163-1 (5), GTR1-1 (6), GTR1-3 (7) were used. GVB isolate 94/971 (Goszczynski et al., 1996) (8) and buffer (9) were also used as additional control of RT-PCR specificity. M = molecular weight marker VIII (Roche).

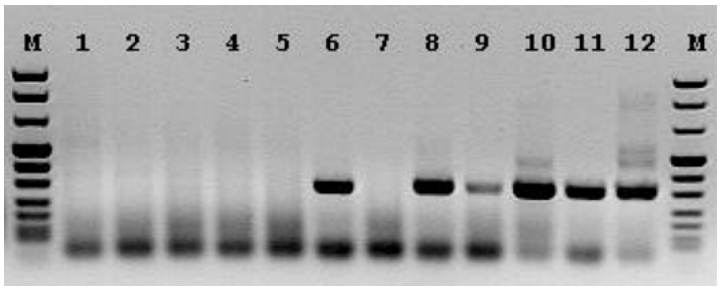

Figure 4. RT-PCR detection of GVA from various grapevines using oligonucleotide primers for specific detection of the virus variants of group III. Grapevines, which were tested GVA-negative (1–5) and strongly positive (6–12) in RT-PCR using oligonucleotide primers for simultaneous detection of all isolates of the virus were used in the experiment. M = molecular weight marker VIII (Roche).

for isolates P163-1, GTR1-1 and GTR1-3 (Figure 3B). No PCR product was observed when dsRNA of isolates GTG11-1, 92/778, JP98 or MSH18-1 were tested using the above primers, confirming that these isolates did not contain GVA variants of group III. The lack of RT-PCR amplification of dsRNA isolated from *N. benthamiana* infected with GVB, or the buffer alone, also indicated specificity of this RT-PCR. Surprisingly, from the selected seven grapevines testing strongly positive for GVA in RT-PCR using primers for simultaneous detection of all GVA variants (not shown), six tested clearly positive in RT-PCR using primers for specific detection of the virus variants of group III (Figure 4). Only one grapevine tested consistently negative for this group of variants, which strongly suggests that these variants are common in vineyards and frequently occur together with other variants of the virus. The findings of this study should be taken into account in future attempts to study the involvement of GVA in grapevine diseases.

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