CERTIFICATION PROTOCOL FOR THE GRAPEVINE LEAFROLL-ASSOCIATED VIRUS TYPE 1 (GLRaV-1)



FINAL REPORT to

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1. Abstract:

The 17,647 nucleotide genome sequence of GLRaV-1 has now been completed and sequence data analysed using bioinformatics. The shortcomings of the current tests for leafroll detection were addressed. The current RT-PCR test was inadequate due to poor reliability and reproducibility of results. The GLRaV-1 RT-PCR certification protocol has been improved by using magnetic capture hybridisation prior to RT-PCR. The MCH-RT-PCR certification protocol has been demonstrated to provide a higher level of sensitivity and reproducibility than the current GLRaV-1 certification protocol used by the Australian industry. The MCH-RT-PCR technique provides an effective and practical screen to identify grapevine samples infected with GLRaV-1. The same protocol is applicable to other grapevine viruses.

2. Executive summary:

Candidate grapevine samples have been maintained allowing the processing of sufficient material for the project. High quality viral double stranded RNA has been purified from infected plant material and used to perform RT-PCR and 5' RACE on the 5' end of the viral genome. The 17,647 nucleotide genome sequence of GLRaV-1 has now been completed and sequence data analysed using bioinformatics.

The shortcomings of the current tests for leafroll detection were addressed. The current RT-PCR test was inadequate due to poor reliability and reproducibility of results, presumably due to sequence variation within the virus and inhibition from excess RNA and impurities such as phenolics.

The method for purifying the viral RNA has been optimised using magnetic capture hybridisation (MCH) prior to RT-PCR. This process removes any potential RT-PCR inhibitors from the sample, therefore improving reliability. This procedure also concentrates the RNA allowing addition of at least 100 fold more RNA into the reaction, therefore increasing sensitivity. The problem of RT-PCR reliability associated with viral sequence variation has been removed by targeting primers to ORF9, a highly conserved region of the genome. This has also provided the benefit of targeting a region of the viral genome with high copy number due to the presence of ORF9 on all 3' subgenomic RNAs.

The magnetic capture RT-PCR technique has been tested on a range of different grapevine varieties from the field. 28 samples were screened for the presence of GLRaV-1 showing 9 positives using the current RT-PCR and ELISA tests. The use of magnetic capture prior to RT-PCR allowed the detection of 16 positive samples. The MCH-RT-PCR technique provides an effective and practical screen to identify grapevine samples infected with GLRaV-1.

Parallel to this research, further experiments aimed at characterising proteins encoded by GLRaV-1 have been completed. The localisation pattern of a protein with a cell can suggest possible roles for the gene in the virus life cycle. Studies involving ORF fusions with green fluorescent protein have highlighted a number of possible interesting protein functions for future research including ORFs 2, 3, 6 and 9 which seem to be involved in virus movement and maintenance. ORF2 appears to alter the internal structure of the cell while ORF9 is targeted to the nucleus suggesting both viral

proteins are interacting with plant host factors. These results have been repeated in planta using a virus-based vector and analysed using confocal microscopy and transmission electron microscopy.

All project milestones and objectives have been achieved and the research is now being put in writing for two publications in scientific journals.

3. Background:

Leafroll is one of the most important viral diseases in Australia and among seven viruses associated with this disease, Grapevine leafroll-associated virus type 1 (GLRaV-1) is the most damaging, causing both the loss of yield and quality as well as being involved in a range of graft incapability problems. For these reasons, GLRaV-1 is the main subject of a strict screening program conducted annually by AVIA. As the main laboratory worldwide studying GLRaV-1, we have developed the current detection procedure in use by the industry. However, observations in our laboratory and by colleagues overseas have raised the issue that some GLRaV-1 infected vines can go through the test undetected. To resolve this, we have completed a study and found that GLRaV-1 has an unusual level of variation in its genome structure, to the extent that it appears like a family of related viruses rather than a discrete species. This has a significant implication for the detection of infected vines.

The original objectives of this study were to:

- Analyze the variability of GLRaV-1 in the field
- Assess the current detection protocols in respect to an unusual virus variation observed
- Complete the genome analysis of GLRaV-1
- Produce an appropriate certification protocol

4. Project Aims and Performance targets:

	Original Outputs	Performance Target
1	Appoint Ph.D. student	July 2000
2	Select candidate infected varieties for the study, collect and process samples for storage	December 2000
3	Prepare the dsRNA and perform RT-PCR on the 5' end of GLRaV-1 genome	December 2001
4	Clone the RT-PCR product and sequence at least 5 clones for each region	December 2001
5	Complete the sequence of GLRaV-1 and analyze the sequence data	December 2001
6	Isolate dsRNA from a range of GLRaV-1 infected vines	July 2002
7	Screen for virus by ELISA	December 2002
8	Use the same material to test detection with a range of primers scanning the genome	July 2003
9	Optimize the detection protocol for detection across all of the varieties collected	July 2003
10	Write papers for scientific/industry journals and communicate findings with industry	July 2003

This project contained two main objectives; to complete the genome sequence of Grapevine leafroll-associated virus type 1 and to develop an appropriate GLRaV-1 certification protocol. Whilst the second objective was met successfully on time, major technical difficulties delayed the completion of sequencing. In order to determine the full sequence of GLRaV-1, a stock of high quality virus double-stranded RNA (dsRNA) was purified from infected plant material. The dsRNA was then used as a template for reverse transcription PCR (RT-PCR), and the products cloned and sequenced. Optimisation of this process allowed the completion of the genome sequence to the 5' end. This delay resulted in the need to extend the project completion date by six months.

5. Method:

Viral dsRNA was extracted from green bark tissues of symptomatic plants according to Rezaian *et al* (1991). Samples of dsRNA were analyzed by electrophoresis in 6% polyacrylamide gel in TAE buffer and visualized by silver staining.

DNA clones were initially synthesized from purified dsRNA by a protocol developed for grapevine leafroll-associated viruses (Fazeli *et al*, 1998). Random hexamer linked to a known oligonucleotide at its 5'-end was used to synthesize single-stranded DNA representing both strands of the dsRNA. The excess primer was removed and double-stranded DNA amplified by PCR. The PCR products were ligated into a pGEM-T vector and electroporated into *E. coli* strain XL1-Blue. Recombinant plasmids were purified and sequenced by automated cycle sequencing. The sequence information was used for developing improved protocols for virus identification and certification. As mentioned in section 4, the procedure involved in generating GLRaV-1 partial clones did not provide sufficient sequence information from the 5' end of the virus genome. This optimization required using adapter ligation instead of random hexamers for the synthesis of the second strand prior to PCR amplification. This technique allowed the rapid completion of the GLRaV-1 genome sequence.

Various grapevine samples were collected with suspicion of leafroll symptoms. Each sample was tested for the presence of GLRaV-1 with the current ELISA and RT-PCR certification protocols. The samples were also screened using primers targeted to different regions of the GLRaV-1 genome. Improvements were made to the procedure used to purify the viral RNA from the plant tissue. This involved the use of magnetic capture hybridisation prior to RT-PCR. Initially developed for the detection of bacterial DNA from soil (Jacobsen, 1995), magnetic capture is a very useful tool for the separation of specific target nucleic acid from other nucleic acids and from interfering compounds.

6. Results/Discussion:

The complete results with detailed discussion are presented in the form of scientific papers to be submitted for publication in refereed journals. The manuscripts are attached in Appendix 1.

7. Outcome/Conclusion:

All project milestones and objectives have been achieved and the research is now being put in writing for two publications in scientific journals.

Reliable detection of GLRaV-1 will be a benefit to the industry reducing the risk of distributing GLRaV-1 infected material and reducing the risk of incompatibility with grafted vines and in the event of top working. The trend for the use of an increasing number of grafted vines has created a need for a more thorough testing of both rootstock and scion materials. This project will help in minimizing the risk of mixing GLRaV-1 with other viruses and thus avoiding graft incompatibility problems.

The completion of the GLRaV-1 genome sequence has opened up a range of new research opportunities with potential benefits to both the scientific community and the grapevine industry.

8. Recommendations:

The etiology of grapevine leafroll disease has not been proven previously due to the need to fulfil Koch's postulates, hence GLRaV-1 is said to be associated with leafroll disease. To satisfy Koch's postulates the suspected disease agent must be purified from a diseased plant and must cause the same disease when introduced into an otherwise healthy body. This cannot be achieved without a full length infectious clone of GLRaV-1, which is now possible due to the completion of the GLRaV-1 genome sequence. The construction of a full-length infectious clone of GLRaV-1 would be a world first generating interest from the international community of plant virologists. It would be a significant outcome of a future project and would benefit both the scientific field and the industry. It would also open up a broad range of research opportunities into GLRaV-1. For example, giving the ability to:

1. Further analyse GLRaV-1 infection at the cellular level.
2. Determine the effects of GLRaV-1 infection on wine quality in various grapevine cultivars.
3. Of most importance to the grapevine industry is the opportunity to create a virus expression vector using GLRaV-1.

The construction of a virus vector containing the minimal genes required for replication would provide a powerful tool to express foreign proteins in grapevines without genetic modification processes. There are currently no viral expression vector systems designed for expression in grapevines. The use of a virus expression system could significantly decrease the time and cost required for such research. This application of GLRaV-1 would aid research in a wide range of grapevine projects, which would be invaluable to the grapevine industry.

9. Appendix 1: Communication:

The results of this project have been presented at national and international conferences. Manuscripts have been prepared for publication in peer-reviewed journals in the area of virology and plant science.

Journal articles

Little A, and Rezaian M. A. (2004). Subcellular localisation of proteins encoded by the Grapevine leafroll-associated virus 1 genome reveals a vesicle inducing protein. To be submitted to Virology.

Little A, and Rezaian M. A. (2004). Improved detection of Grapevine leafroll-associated virus 1 by magnetic capture hybridisation RT-PCR. To be submitted to the Journal of Virological Methods.

Conference papers

Little, A. and Rezaian, M. A. Completed sequence, improved detection and gene functional analysis of Grapevine leafroll-associated virus 1. Oral presentation at the 2nd Australian Virology Group meeting, Fraser Island, Australia, December 2nd-6th 2003.

Little, A. and Rezaian, M. A. Completed sequence, improved detection and gene functional analysis of Grapevine leafroll-associated virus 1. Oral presentation at the 14th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Bari, Italy, September 12th-17th 2003.

Little, A., Fazeli, C. F. and Rezaian, M. A. Hypervariable genes in Grapevine leafroll-associated virus 1. Poster presentation at the 13th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Adelaide, Australia, March 12th-17th 2000.

Little, A., Fazeli, C. F. and Rezaian, M. A. Hypervariable genes in Grapevine leafroll-associated virus 1. Poster presentation at the 1st Australian Virology Group meeting, Fraser Island, Australia, December 5th-9th 2001.

10. Appendix 2: Intellectual Property:

11. Appendix 3: References

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12. Appendix 4: Staff

Name	Title	Position	Qualification	% of time to be spent on the project

Alan Little		Ph.D. student	Honours	100%
Ali Rezaian	Dr.	Senior Scientist	Ph.D.	20%

Improved detection of Grapevine leafroll-associated virus 1 by magnetic capture hybridisation RT-PCR.

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Abstract
We report the development of a sensitive diagnostic method for the detection of the Grapevine leafroll-asociated virus 1 (GLRaV-1). We have considered the current shortcoming in detection of GLRaV-1 to be linked to two factors, variability in the viral genome and low template concentration. Analysis of the GLRaV-1 genome has provided optimal target regions for detection by RT-PCR with high copy number and low levels of sequence variation. This was combined with the use of Magnetic Capture Hybridisation RT-PCR to allow the removal of RT-PCR inhibitors and the addition of 100-fold template RNA to a single RT-PCR. The reproducibility of the technique was confirmed using field samples.

Keywords
grapevine, leafroll, GLRaV-1, magnetic capture.

Introduction
Leafroll is an important disease of grapevines causing crop loss, reduced sugar content of fruit, delayed fruit maturity, abnormal leaf coloration and rolling of leaves (Goheen, 1970, Bovey *et al*., 1980, Bovey and Martelli, 1992). Several serologically distinct members of the family *Closteroviridae* are associated with Leafroll disease. Grapevine leafroll-associated virus 1 (GLRaV-1) is one of the most important types worldwide. It is present in some of the major grapevine varieties grown in Australia and is associated with low crop yields in Sultana clones (Habili *et al*., 1996, 1997). Apart from transmission via vegetative propagation and grafting, GLRaV-1 may be transmitted by the scale insect *Parthenolecanium corni* and by the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza *et al*., 2000).

The 17,647 nucleotide sequence of GLRaV-1 has recently been completed (Little and Rezaian, 2004; Fazeli and Rezaian, 2000). The overall organisation of the GLRaV-1 genome is similar to those of other members of the family *Closteroviridae* (Dolja *et al*., 1994) and it is most closely related to GLRaV-3 (Fazeli and Rezaian, 2000). The GLRaV-1 genome contains 9 open reading frames encoding in the 5' to 3' direction the putative replication complex consisting of the methyltransferase (MTR), helicase (HEL) and RNA dependent RNA polymerase (RdRp) domains, a small hydrophobic protein, a heat shock protein 70 (HSP70) homologue, a HSP90-like protein, the coat protein (CP), two diverged copies of the CP (CPd1 and CPd2) and two other proteins of unknown function. Unlike other members of the family *Closteroviridae*, the duplication of the GLRaV-1 CP gene occurs in two ORFs.

The use of grapevine propagation material certified negative for GLRaV-1 is a common practice worldwide. The International Council for the study of Virus and Virus-like Diseases of the Grapevine (ICVG) recommends that in order to preserve valuable grape clones and varieties, certified selections should be tested for a list of specific pathogens including GLRaV-1. Australia, conducts large scale screening of planting material with the aim of eliminating GLRaV-1 from the field. These tests are commonly carried out by PCR that offers speed and low cost compared to biological indexing. However, there are serious shortcomings regarding reproducibility of these tests (GWRDC, 2002).

In an attempt to improve the consistency of these tests, the level of sequence heterogeneity in the GLRaV-1 genome was determined (Little *et al*., 2001). Sequence variation across the genome was not uniform, showing clustering mainly in ORFs 3, 6 and 7 corresponding to the HSP70-like protein and CPd1 and CPd2 respectively. Overall, 75 clones corresponding to ORFs 3, 6 and 7 were sequenced and 1916 nucleotide changes were recorded relative to the published sequence. None of the changes resulted in a frame shift or stop codon and there was a trend for the conservation of amino acids or change to amino acids having similar physiochemical properties. The CPd2 gene was particularly variable with a mutation seen in 60% of the nucleotide positions in the cDNA clones sequenced. These observations suggest that GLRaV-1 may exist in the form of a heterogeneous population, possibly resulting from the lack of selective pressure and from mixing of virus strains due to viticulture practices of vegetative propagation and grafting over the centuries.

In Australia, the region targeted for detection by RT-PCR in the current industry certification protocol is ORF7, which could contribute the inconsistency of tests. It is believed that variable results in grapevines are due to tissue specificity and viral titres in them, and phenolic content. In this report we present the development of diagnostic method for GLRaV-1 in grapevines, which deals with the shortcomings in detection due to variability in the viral genome and sensitivity of detection.

Methods

Preparation of Total RNA for GLRaV-1 detection

Total RNA was isolated from green bark tissue using a sodium perchlorate method (Rezaian and Krake 1987) with modifications. Plant material, 1g, was ground to a powder in liquid nitrogen with a pre-cooled mortar and pestle. Tissue samples were extracted in 20ml of RNA extraction buffer [5M sodium perchlorate, 0.3M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) ßmercaptoethanol, 8.5% (w/v) PVPP, 2% (w/v) PEG 4000] and homogenized by shaking at room temperature for 30min. The homogenate was transferred to a crude separation apparatus consisting of a 20ml disposable syringe plugged with silane-treated glass wool wrapped in a double layer of Miracloth. The syringe was secured in a 250ml centrifuge tube (Corning, Acton, USA) and then centrifuged at 400g for 10min at 4°C though. 15ml of modified extraction buffer [5M sodium perchlorate, 0.3M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) ßmercaptoethanol] was added to the syringe and centrifuged as before. The combined eluate was centrifuged at 5000g for 10min at 4°C. The supernatant was removed, mixed with 2.5 volumes of cold ethanol, stored at -20°C for a minimum of 1 hour and then centrifuged at 5000g for 20min at 4°C. The resulting pellet was dried under vacuum and resuspended in 1ml of 10mM Tris-EDTA (pH 7.6). The suspension was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). The upper aqueous phase was retained, mixed with 2.5 volumes of cold ethanol and 0.1 volumes of sodium acetate pH 5.2 and stored at -20°C overnight. The RNA precipitate was pelleted by centrifugation at 7700g for 10min at 4°C, dried under vacuum, rinsed with cold 70% ethanol, resuspended in 200ml of cold sterile water, and stored at -80°C. The concentration of the RNA was determined spectrophotometrically. Small scale total RNA extraction from 100mg green bark tissue was performed using a modified Rneasy Plant mini kit (QIAGEN, Hilden, Germany) (MacKenzie *et al*., 1997).

Design and Synthesis of biotin-labelled capture probe

An 80-nt oligonucleotide complementary to the 3'-end of the GLRaV-1 RNA genome (nucleotides 17567 to 17647) was designed. The capture sequence was designed 83-nt downstream of the 3'-terminal nucleotide of ORF9, ending at nucleotide position 17484, to avoid binding competition during subsequent RT-PCR tests. The capture oligonucleotide incorporated a biotin molecule on twelve-atom triethylene glycol linker and was synthesised by Geneworks PTY LTD, Australia.

Preparation of magnetic particles

The Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) in 0.6ml aliquots (Promega) were completely dispersed by flicking and captured by placing the tube in a magnetic stand for approximately 30 seconds. The supernatant was removed and the particles were washed three times with 300µl of 0.5xSSC. The particles were resuspended in 100µl of 0.5xSSC ready for use.

Hybridisation of capture probe and viral RNA

Samples of total RNA extracted from 500mg green bark tissue were combined with 3µl of 50µM biotinylated oligonucleotide in a final volume of 500µl. The mixture was incubated at 65° for 10min, 13µl of 20xSSC was added and allowed to cool at room temperature. The entire contents of the annealing reaction was added to the washed SA-PMPs and incubated at room temperature for 10min on a spinning rotor. The SA-PMPs were collected, the supernatant removed and the particles were washed four times in 0.2x SSC and eluted twice in 250µl sterile water. The elutions were pooled for each sample and viral RNA stored at -80°.

Single tube RT-PCR

Samples were synthesised with the Superscript One-Step RT-PCR Kit (Promega) in a 50µl reaction using GLRaV-1 specific oligonucleotides pORF9F and pORF9R (GGC TCG AGA TGG CGT CAC TTA TAC CTA and CCT CTA GAC ACC AAA TTG CTA GCG A respectively), which amplify a 633-bp DNA fragment. The RT-PCR protocol consisted of 30 min at 50°, followed by 40 cycles of 15 sec at 94°, 30sec at 55°, 1min at 68°, and finally an extension time of 10min at 72°. The RT-PCR

reaction was analysed on a 1% agarose/EtBr gel run in 1xTBE buffer. A 1kb Plus DNA ladder (Gibco, BRL) was used as a size marker.

Results and Discussion

Optimised target region for GLRaV-1 diagnosis

To achieve optimal detection of GLRaV-1 by RT-PCR it was necessary to target the viral RNA region with low levels of sequence variation. Using the sequence information available (Little *et al*., 2001), we tested regions of low variability within ORFs 1b, 5 and 9 as targets. 19 samples suspected of leafroll disease infection were collected from the field. Each sample was tested for the presence of GLRaV-1 by ELISA targeted to the coat protein (ORF5) prior to RT-PCR tests (Fig. 1). 7 samples gave a positive result with ELISA. Only 4 samples were positive using primers to ORF1b, primers targeted to ORF5 gave 6 positives and 12 samples were positive using primers targeted to ORF9. The detection levels were the lowest at the 5' end of the viral genome and highest at the 3' end. This is most likely due to the increased copy number of GLRaV-1 ORFs at the 3' end of the genome due to the presence of sub-genomic RNAs (Fig. 2). GLRaV-1 encodes three sub-genomic promoters across its genome. The first sub-genomic RNA promoter is positioned upstream of ORF5, which would explain the increase in detection using primers targeted to ORF5 compared to ORF1b. The second sub-genomic promoter is positioned upstream of ORF8 and the third is upstream of ORF9. This accounts for the dramatic increase in sensitivity seen when using primers targeted to ORF9 relative to ORF1b and ORF5. ORF9 was therefore considered as a suitable candidate for detection of GLRaV-1 by RT-PCR because it is present in all 3' sub-genomic RNAs (Fig. 2) (Fazeli *et al*., 2000), and shows the lowest amount of variation seen for all regions across the genome (Little *et al*., 2001).

Optimised detection of GLRaV-1 using magnetic capture

The presence of phenolic compounds in grapevine extracts interferes with virus detection by enzymatic methods (Minafra and Hadidi, 1994). In an effort to further purify the viral RNA template we used Magnetic Capture Hybridisation in combination with RT-PCR (MCH-RT-PCR). Initially developed for the detection of bacterial DNA from soil (Jacobsen, 1995), magnetic capture allows the separation of specific target nucleic acid from other nucleic acids and from interfering compounds. We obtained a biotinylated oligonucleotide to the 3´ untranslated region present in all GLRaV-1 genomic and sub-genomic RNA species (Fig. 3). Following hybridisation in solution the RNA was captured and the non-specific total RNA and PCR inhibitors were washed away. This procedure yields an essentially pure fraction of GLRaV-1 RNA after only a single round of magnetic separation. The grapevine samples can also be freeze dried to concentrate the viral RNA prior to RT-PCR.

The magnetic capture technique was tested using a GLRaV-1 infected sample which produced inconclusive results when tested by RT-PCR giving a positive result using primers for ORF9 and a negative result using primers for ORF1b and ORF5. The total RNA was extracted from 1g of the grapevine sample and split into two aliquots. Half was left as total RNA and the other half was purified using magnetic capture (MC-RNA). RT-PCR of ORF9 was performed using total RNA and MC-RNA as templates (Fig. 4). Each reaction contained the equivalent of tissue weights ranging from 0.1mg to 100mg. RT-PCR using total RNA gave a positive result using 1mg, however a negative result occurred at lower and higher tissue concentrations (Fig. 4a). At the lower tissue input of 0.1mg the reaction was not sensitive enough for detection. However, when more tissue was added to the reaction then a false negative occurred presumably due to inhibition from excess RNA and impurities such as known to be polyphenolics (Koonjul *et al*., 1999). A strong positive result was obtained when performing RT-PCR using magnetic captured RNA to the equivalent of 100mg tissue (Fig. 4b). This gave the equivalent of adding 100-fold more viral RNA into a single RT-PCR reaction. The higher viral template concentration improved the sensitivity of the certification protocol while the increased purity removed the potential for false negatives due to the presence of PCR inhibitors.

Evaluation of MCH-RT-PCR using field material

To assess the practical reproducibility of MCH-RT-PCR with field material, 28 grapevine samples were collected from the field. These samples were suspected of leafroll disease infection. Each sample was tested for the presence of GLRaV-1 using RT-PCR targeted to ORF7 and ELISA targeted to ORF5 (Table 1). Nine samples tested positive by both methods. The change of RT-PCR primers from the variable ORF7 to the more conserved ORF9 was tested resulting in an increase of the number of positive samples from 9 to 14. However, with magnetic capture RT-PCR 16 positives were detected from the sample group. The current certification protocol would have detected only 56% of the infected samples detected by MCH-RT-PCR.

In conclusion, we have combined the knowledge of sequence variation in GLRaV-1 and magnetic capture to devise an improved diagnostic for Grapevine leafroll-associated virus 1. This approach could be adapted to other grapevine viruses were detection is limited by low viral template and high levels of PCR inhibitors.

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Figure Legends

Fig. 1. Diagram indicating the regions of the GLRaV-1 genome targeted for detection by RT-PCR. Results obtained from screen of suspected GLRaV-1 infected grapevine samples using ELISA and RT-PCR targeted to ORFs 1b, 5 and 9.

Fig. 2. Genome organisation of GLRaV-1 and sub-genomic RNAs. Rectangles represent ORFs. P-Pro, papain-like protease; MTR, methyltransferase; HEL, helicase; POL, polymerase; HSP70, homologue of HSP70 proteins; CP, coat protein; CPd, diverged copy of coat protein.

Fig. 3. Schematic diagram of GLRaV-1 RNA isolation using magnetic capture with paramagnetic particles (PMP).

Fig. 4. Improved sensitivity and reliability of MCH-RT-PCR for detection of GLRaV-1 in RNA preparations from grapevine tissue. (a) RT-PCR without MCH; Lane 1, negative control. Lane 2, DNA positive control using 1ng pGEM-ORF9. Lanes 3-6, Healthy sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of total RNA respectively. Lanes 7-10, Infected sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of total RNA respectively. (b) RT-PCR with MCH; Lane 1, negative control. Lane 2, DNA positive control using 1ng pGEM-ORF9. Lanes 3-6, Healthy sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of MC RNA respectively. Lanes 7-10, Infected sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of MC RNA respectively.

Figure 1

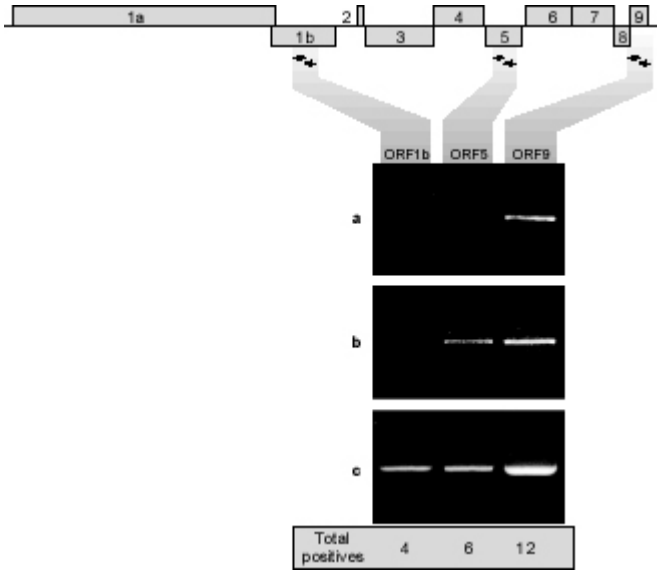


Figure 2

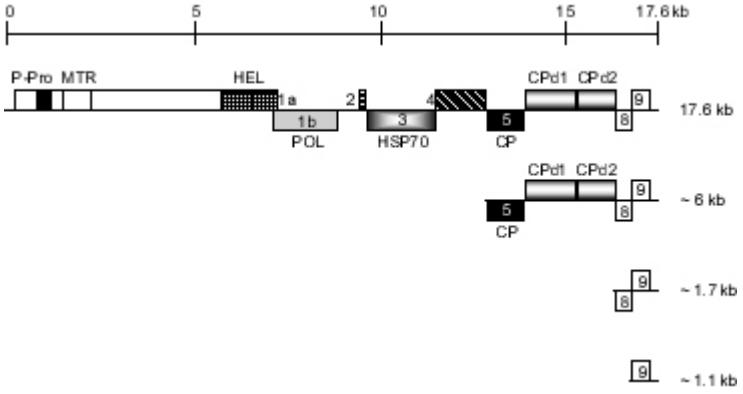


Figure 3

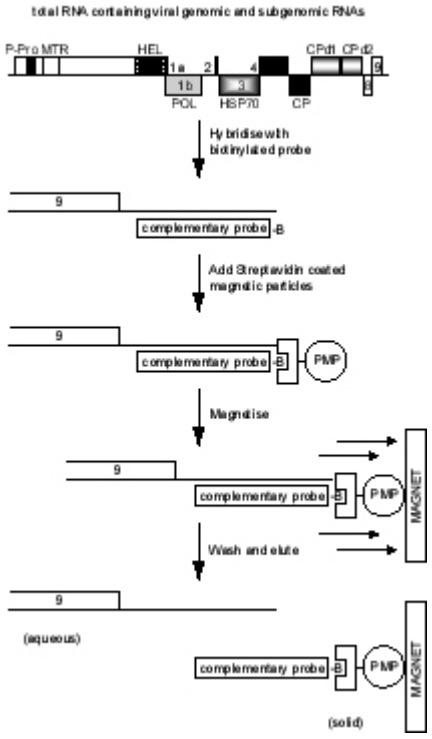


Figure 4


(a)
DNA control
Healthy
Infected
- + 0.1 1 10 100 0.1 1 10 100
(mg) Tissue equivalent
633nt (ORF9)
(b)
DNA control
Healthy
Infected
- + 0.1 1 10 100 0.1 1 10 100
(mg) Tissue equivalent
633nt (ORF9)

Subcellular Localisation of Proteins Encoded by the Grapevine Leafroll-associated Virus 1 Genome Reveals a Vesicle Inducing Protein

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Abstract

The sequence of 5,252 nucleotides (nt) from the 5'-end of the Grapevine leafroll-associated virus 1 (GLRaV-1) was determined, completing the 17,647-nt genome sequence of the virus genome. The 5'-terminal sequence contains overlapping open- reading frames (ORFs) 1a and 1b, encoding the papain-like proteinase, methyltransferase, RNA helicase and RNA-dependent RNA polymerase domains. We report the observed subcellular localisation patterns of all putative proteins encoded by the GLRaV-1 genome, except the ORF1a polyprotein. Using gene fusions to the green fluorescent protein, four distinct patterns of localisation were observed when expressed in onion epidermal cell layers and in *Nicotiana tabacum* leaves. ORF3 and ORF6 localised to the cell periphery, ORF9 appeared to be localized to the nucleus and congregated within the nucleolus and ORF2 showed association with punctate bodies of various sizes. Our results indicate that ORF2 is the viral encoded protein associated with the formation of multivesicular bodies that appear to originate from the endoplasmic reticulum.

Keywords

closterovirus, ampelovirus, crinivirus, closteroviridae, grapevine, leafroll, GLRaV-1.

Introduction

Leafroll is an important disease of grapevines causing crop loss, reduced sugar content of fruit, delayed fruit maturity, abnormal leaf coloration and rolling of leaves (Goheen, 1970, Bovey *et al*., 1980, Bovey and Martelli, 1992). Several serologically distinct members of the family *Closteroviridae* are associated with Leafroll disease. Grapevine leafroll-associated virus 1 (GLRaV-1) is one of the most important types. It is present in some of the major grapevine varieties grown in Australia and is associated with low crop yields in Sultana clones (Habili *et al*., 1996, 1997). Apart from transmission via vegetative propagation and grafting, GLRaV-1 may be spread by the scale insect *Parthenolecanium corni* and by the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza *et al*., 2000).

The nucleotide sequence of the 3' 12,395-nt of the GLRaV-1 genome has been previously determined (Fazeli and Rezaian, 2000). The overall organisation of the GLRaV-1 genome is similar to those of other members of the family *Closteroviridae* (Dolja et al., 1994) and is most closely related to GLRaV-3 (Fazeli and Rezaian, 2000). The 3' region of GLRaV-1 genome contains 8 open reading frames (ORF) encoding in the 5' to 3' direction a small hydrophobic protein, a heat shock protein 70 (HSP70) homologue, a HSP90-like protein, the coat protein (CP), two diverged copies of the CP (CPd1 and CPd2) and two other proteins of unknown function. Unlike other members of the family *Closteroviridae*, the duplication of the GLRaV-1 CP gene occurs in two ORFs. In this paper, we report the complete 17,647-nt sequence of the GLRaV-1 genome. The 5' region of the genome encodes the putative replicase consisting of the methyltransferase (MTR), helicase (HEL) and RNA dependent RNA polymerase (RdRp) domains, with the latter domain likely to be expressed via a +1 ribosomal frameshift.

The family *Closteroviridae* comprises the *Ampelovirus*, *Closterovirus* and *Crinivirus* genera. The function of *Closteroviridae* proteins has been analysed for the *Closterovirus* type member *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV) due to the availability of full length infectious clones. This has allowed the use of knockout constructs to determine the genes essential for viral replication and movement. Subcellular localisation studies have provided further evidence for the suggested roles of individual proteins, which were previously assigned functions based on sequence alignments with other viruses in the 'Sinbis-like' supergroup of positive-strand RNA viruses. In this study, we report the observed subcellular localisation patterns of all the GLRaV-1 open reading frame (ORF) products, except the ORF1a polyprotein, using gene fusions to the green fluorescent protein (GFP).

Methods

Isolation and analysis of GLRaV-1 dsRNA

Viral dsRNA was extracted from green bark tissue of GLRaV-1-infected Sultana, clone B4L (Habili *et al*., 1997; Woodham *et al*., 1984) by the method of Rezaian *et al*., (1991). Samples were analysed by electrophoresis in a 6% polyacrylamide gel in TAE buffer (Loening, 1967) and visualised by silver staining (Merril *et al*., 1981).

Synthesis of GLRaV-1 specific dsDNA

First strand cDNA was synthesised using a GLRaV-1 specific primer designed to the most 5' previously known sequence. Samples of dsRNA extracted from 5g green bark tissue of GLRaV-1 infected grapevine were combined with 1µl of 10µM specific primer in a final volume of 5µl, boiled for 5 minutes and cooled on ice for 2 minutes. Reverse transcription was carried out for 1½ hours at 37° in a 10µl reaction containing 50mM Tris-HCl (pH 8.5), 8mm $MgCl_2$, 30mM KCl, 1mM DTT, 1mM each of the four dNTPs and 20 units of AMV reverse transcriptase (Clontech, Palo Alto). Second strand DNA synthesis was carried out for 2 hours at 16° in a 50µl reaction by adding the second strand reaction mix containing 100mM KCl, 10mM ammonium sulphate, 5mM $MgCl_2$, 0.15mM β-NAD, 20mM Tris-HCl (pH 7.5), 0.5mg/ml BSA, 0.3mM each of the four dNTPs, 0.3 units of DNA polymerase I, 0.0125 units of RNase H and 0.06 units of *E. coli* DNA ligase (Clontech, Palo Alto). After addition of 2µl of T4 DNA polymerase (3 units/µl) the reaction was incubated for 30min at 16°C to blunt the DNA ends. The reaction was terminated by the addition of EDTA and glycogen to the final concentrations of 0.1mM and 50ng/ml respectively. After a chloroform:isoamyl alcohol (24:1) extraction, the DNA was ethanol precipitated using ultracentrifugation at 250,000g for 1 hour in a swing-out rotor. Samples were resuspended in 5µl of nuclease-free water and 2µl of 10µM Adaptor #1 from the Clontech cDNA subtraction kit (Cat #K1804-1) was added. Adaptor ligation was carried out overnight at 16°C in a final volume of 10µl containing 50mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 2mM DTT, 0.05mg/ml BSA and 40units of T4 DNA ligase (Clontech, Palo Alto). The reaction was incubated for 5min at 72°C to inactivate the enzyme. PCR was performed using the specific oligonucleotide originally used for cDNA synthesis and an oligonucleotide targeted to adaptor #1. The reaction in 25µl contained 40mM Tris-HCl (pH 9.2), 15mM KOAc, 3.5mM $Mg(OAc)_2$, 3.75µg/ml BSA, 0.2mM of each dNTP, 1x Advantage cDNA polymerase mix (Clontech, Palo Alto) and 1ul of a 1/200 dilution of the ligation reaction. The PCR protocol consisted of 5 min at 75°, followed by 20 cycles of 30 sec at 94°, 30 sec at 65°, 4 min at 68°, and finally an extension time of 10 min at 68°. The PCR reaction was analysed on a 1% agarose/EtBr gel run in 1x TBE buffer. A 1kb Plus DNA ladder (Gibco, BRL, Gaithersburg) was used as a size marker.

cDNA cloning and sequencing

The PCR products were run on a 1% agarose gel and products in a size range between 1000 and 4000-nt were gel extracted and ligated into a pGEM-T Easy vector (Promega, Southampton). Recombinant plasmids were released by digestion with *Eco*RI and run on a 1% agarose gel in TBE to determine insert size. The largest positive clones were sequenced by PCR using the dideoxynucleotide chain termination method (Applied Biosystems model 377 DNA sequencer) with double stranded plasmid templates and universal sequencing primers M13 and SP6 (Stratagene, La Jolla).

Sequence data were analysed using the GCG package (Genetics Computer Group, USA). Searching of the non-redundant amino acid sequence database of the National Center for Biotechnology Information (NCBI) was performed using the programs BLAST and BLASTP (Altschul *et al*., 1990). A helical wheel diagram of the 25-40 amino acid region of ORF2 was produced using the WHEEL program (Jones et al., 1992).

5'-end sequencing by the RACE procedure

Clones representative of 5'-termini of the GLRaV-1 genome were obtained using a GeneRacer Kit (Invitrogen, Carlsbad) according to the manufacturer's instructions. Briefly, dsRNA extracted from 5g green bark tissue of GLRaV-1 infected grapevine were combined 2 µg of the control total RNA supplied with the kit and treated with calf intestinal alkaline phosphatase and subsequently with tobacco acid pyrophosphatase. This procedure is designed to leave a 5'-phosphate moiety only on RNAs that contain a cap structure, thus selecting for 5'-full length RNA. The RNAs were joined to the Gene Racer 5'-RNA oligo (5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA) using RNA ligase. The ligated mRNA was reverse transcribed using Superscript II reverse transcriptase and a gene-specific primer, 5'- CAGACTCCAATTCCACAACGCTGG (reverse complement of bases 208 to 231). The initial PCR utilized GeneRacer 5'-primer (5'-CGACTGGAGCACGAGGACACTGA) and the gene-specific primer. Nested PCR, when performed, utilised GeneRacer 5'-nested primer (5'-GGACACTGACATGGACTGAAGGAGTA) and 5'- ATTAACCAGACGCACCGTCCCAGA as gene

specific nested primer (reverse complement of bases 183 to 206). The clones were sequenced as outlined above.

GFP fusion constructs

A variant of the shuttle vector pART7 (Gleave, 1992), containing the full-length GFP ORF downstream of the multiple cloning site, without a start codon but containing a stop codon (T. Franks, unpublished data), was used to transiently express GLRaV-1 ORF:GFP fusion proteins from a CaMV 35S promoter. Primers were designed for ORFs 1b to 9 with the forward primer containing an *Xba*I site and a start codon. The reverse primer contained an *Xho*I site with no stop codon allowing an in frame C-terminal fusion with the GFP ORF. The *Not*I-digested fragments from pART7 containing the cauliflower mosaic virus (CaMV)-derived 35S promoter, the GLRaV-1 ORF, and the nopaline synthase (NOS) transcription terminator sequence were then transferred into a binary vector pART27 (Gleave, 1992) to generate pART27:ORF:GFP.

Biolistic Bombardment

To coat tungsten with plasmid DNA, 8mg of particles in 100µl of ethanol was vortexed for two minutes, spun down for 10 seconds in a microfuge, drained, washed twice with 100µl sterile water, and resuspended in 100µl sterile water. 50µl of this suspension was removed and added to 5µl of 1µg/µl plasmid preparation. While gently vortexing, 20µl of cold 1M spermidine and 50µl 2.5M CaCl2 were added drop wise and the resulting mixture incubated on ice for 10 minutes. The particles were spun down, drained, resuspended in cold ethanol, and placed on sterile filter holders.

Onion epidermal strips were placed on agar containing Murashige and Skoog Salt Mixture (Invitrogen, Carlsbad, California) and bombarded with settings of 620kPa helium pressure after evacuating the chamber to -90kPa. Tissue was stored in the dark for 48 hours, and observed using a Leica stereomicroscope coupled to a fluorescence module.

Agroinfiltration

pART27:ORF:GFP constructs were introduced into *Agrobacterium tumefaciens* (strain C58), which were subsequently introduced into *N. tabacum* leaves (Selth *et al*., 2004). Plant tissue was sampled after five days and checked for GFP expression using a Leica stereomicroscope coupled to a fluorescence module. More detailed images were obtained with a Confocal Laser Scanning Microscope System, Bio-Rad MRC-1000UV, comprised of a Nikon Diaphot 300 inverted microscope and a Krypton/Argon laser. The excitation wavelength used for GFP analysis was 488nm.

Transmission electron microscopy

N. tabacum leaf tissue agroinfiltrated with pART27:ORF2:GFP and pART27:GFP was sampled at 3, 6, 9, 12 and 15 days post infiltration. The tissue was sectioned into 1mm squares and fixed for two to three days in 4% paraformaldehyde/1.25% glutaraldehyde (EM grade) in phosphate buffered saline pH 7.2 (PBS), with 4% sucrose. The tissue was washed twice in PBS and 4% sucrose for 10 minutes and post-fixed in 2% osmium tetroxide for one hour with constant mixing. The tissue was dehydrated sequentially with; 70% ethanol (three changes of twenty minutes each), 90% ethanol (three changes of twenty minutes each), 95% ethanol (three changes of twenty minutes each), 100% ethanol (three changes of twenty minutes each). After a half hour incubation in propylene oxide, resin was infiltrated into each sample overnight at a 1:1 resin:propylene oxide ratio. This was followed by three changes of 100% resin for at least eight hours each with constant mixing. Each sample was embedded into individual moulds and the resin polymerised at 70°C for 24 hours. Thin sections were cut, mounted on an EM copper grid and visualised using a PHILIPS CM100 Transmission Electron Microscope.

Results and Discussion

The complete nucleotide sequence and genome organisation of GLRaV-1

Ten ORFs were identified within the GLRaV-1 genome (Fig. 1). Following the convention used for *Beet yellows virus* (BYV), the type member of the genus *Closterovirus*, these ORFs were designated 1a, 1b and 2 through to 9. The complete 17,647-nt sequence of GLRaV-1 has been submitted to the GenBank database, accession nos. **XXXXX**. The existence of a 5´ cap is postulated from the presence of a methyltransferase region in the replicase gene and the presence of a 5' cap in other members of the family *Closteroviridae*, for example *Beet yellows virus* (Karasev *et al*., 1989). This was further reinforced by the finding that the 5' terminal sequence could only be obtained by 5' RACE using adapter ligation when the viral dsRNA was first treated with tobacco acid pyrophosphatase. This treatment hydrolyses the phosphoric acid anhydride bonds in the triphosphate bridge of the cap structure, releasing the cap nucleoside and generating a 5'-phosphorylated terminus on the RNA molecule.

The GLRaV-1 genome contains a 32-nt 5' untranslated region (UTR) beginning with the sequence GCAAT. This sequence was present in 12 independent DNA clones from this region. The GLRaV-1 5' UTR has no significant sequence similarity to other members of the family *Closteroviridae* and is shorter in length. For example, *Beet yellows virus* and GLRaV-3 have a 5' UTR of 107-nt and 153-nt respectively (Agranovsky *et al*., 1994, Ling, 2003). Computer assisted secondary structure predictions indicated a potential stem-loop structure in the region. This structure spans 26 nucleotides between positions 2 and 27, has a stem of 10 base pairs plus two mismatches, and a loop of 3 nucleotides. This stem-loop is similar to the largest stem-loop structure predicted in the 3' UTR of the GLRaV-1 genome containing 28 nucleotides between positions 17600 and 17628 which includes 12 bonds, one mismatch and a stem-loop of 3 nucleotides (Fazeli and Rezaian, 2000). The significance of these structures in GLRaV-1 during viral replication is unknown. In *Citrus tristeza virus* (CTV), stem-loop structures are conserved in populations with high levels of sequence variability in the 5' UTR (Lopez *et al*., 1998). Most of the variability is accommodated in the loops and, when found in the stems, occurs in such a way that compensatory mutations maintained their general features. This conservation of secondary structure by covariation strongly supports a functional *in vivo* role for it, potentially providing sites for nucleation in RNA folding and for interactions with various proteins (Witherell and Uhlenbeck, 1989).

The initiation codon for ORF1a (positions 33 to 35) represents the most 5' proximal AUG in the sequence and is found in a favourable context, with a U and G at the –3 and +4 positions respectively (Kozak, 1986). The 5' region of the genome contains overlapping ORFs 1a and 1b, encoding products with calculated M_r of 236K and 59K respectively. ORF1b lacks an initiation codon, and based on homology with other members of the family *Closteroviridae* it is predicted that the ORF1b product is expressed via a +1 ribosomal frameshift from the ORF1a/1b transcript. The ORF1a/1b transcript contains the papain-like proteinase (P-Pro), methyltransferase (MTR), RNA helicase (HEL) and RNA-dependent RNA polymerase (RdRp) domains that are conserved in the 'Sindbis-like' supergroup of positive-strand RNA viruses to which the family *Closteroviridae* belongs (Koonin and Dolja, 1993).

The P-Pro domain is located at the 5'-region of ORF1a and was identified by its homology to P-Pro domains from other *Closteroviruses*. The putative catalytic cysteine and histidine residues were at positions 241 and 287 respectively, with the predicted cleavage site occurring between Gly-434 and Gly-435 (Fig. 2). The region of ORF1a directly downstream of the P-Pro domain was identified as an MTR domain based on homology with other members of the family *Closteroviridae* and the six conserved MTR motifs have been identified (Fig. 2) (Rozanov *et al*., 1992).

Phylogenetic analysis of the putative replicase of GLRaV-1 confirm its classification with *Ampeloviruses*

Phylogenetic analysis was performed using the complete amino acid sequences of the putative replicase consisting of the P-Pro, MTR, HEL and RdRp domains of GLRaV-1, *Citrus tristeza virus* (CTV), *Beet yellows virus* (BYV), *Little cherry virus-1* (LChV-1), *Lettuce infectious yellows virus* (LIYV), LChV-2, GLRaV-3, the partially sequenced replicases of GLRaV-2 and Pineapple mealybug wilt-associated virus-2 (PMWaV-2) (Fig. 3). Comparison of these viral sequences showed that they are separated into the genus groups created in the recent revision of the family *Closteroviridae* based on their viral insect vectors and alignment of their HSP70 ORFs (Martelli *et al*., 2002). GLRaV-1 is distant from the aphid-transmissible *Closteroviruses* GLRaV-2, BYV and CTV and the whitefly transmissible *Crinivirus* LIYV. The currently unassigned LChV-1 shows stronger homology to LIYV than in the previous alignment of the HSP70 ORF, strengthening the case for its assignment into the *Crinivirus* group. GLRaV-1 shows strong homology to the other members of the mealybug transmitted *Ampeloviruses* LChV-2, PMWaV-2 and the strongest homology to the type member of the group GLRaV-3 with 31% amino acid sequence identity and 48% sequence similarity (Table 2). This strong relationship with GLRaV-3 is also seen with the HSP70, CP and CPd ORFs (Fazeli and Rezaian, 2000).

Localisation of GLRaV-1 proteins by fluorescence microscopy

The subcellular localisation patterns of all the GLRaV-1 open reading frames (ORFs), except the ORF1a polyprotein, were observed using gene fusions to the green fluorescent protein (GFP). Four distinct patterns of localisation were seen when the fusion constructs were bombarded into onion epidermal cell (Fig. 4). The most common resembled the pattern of untargeted free GFP distributed throughout the cytoplasm and nucleus (Fig 4a). The gene products in this class included the putative RNA-dependent RNA-polymerase (ORF1b, Fig 4b), HSP90 homologue (ORF4, Fig 4e), coat protein (ORF5, Fig 4f), coat protein duplicate #2 (ORF7, Fig 4h) and a protein of unknown function (ORF8, Fig 4i). This pattern of expression is not unexpected given that these proteins are encoded by an RNA virus replicating in the cytoplasm. The RNA dependent RNA polymerase is

expected to target to the replication complex for transcription of the viral genome. The coat protein and coat protein duplicate #2 are also expected to localise close to the replication complex to facilitate the formation of the virion structure following the production of single stranded copies of the viral genome. The lack of specific targeting patterns for these proteins is probably due to the absence of the membrane structures in healthy onion cells where the formation of the viral replication complex is predicted to occur in a normal virus infection process involving coordinated expression of viral genes (Faoro and Carzaniga, 1995).

The HSP70 homologue (ORF3, Fig 4d) and the Coat protein duplicate #1 (ORF6, Fig 4g) were localised to the cell periphery, suggesting roles in cell-cell movement. This is consistent with reports that the HSP70 homologue of BYV was localised to the plasmodesmata (Medina *et al*., 1999) and to function as one of the viral movement proteins (Peremyslov *et al*., 1999). The BYV HSP70 also has a role in chaperoning the assembly of the coat protein duplicate on the tail of the virion (Alzanhova *et al*., 2001). The formation of this tail structure is a prerequisite for BYV cell-cell movement via the plasmodesmata (Alzanhova *et al*., 2001), which is consistent with our observation of GLRaV-1 CPd-1 localisation to the cell periphery. It is unclear why only one of the GLRaV-1 coat protein duplicates is targeted to the cell wall. No comparison can be made with other members of the family *Closteroviridae* because GLRaV-1 is the only member with two coat protein duplicates. This localisation pattern was confirmed *in planta* using agroinfiltration of the fusion constructs in a binary vector into *N. tabacum* and confocal laser scanning microscopy (Fig. 5d and e respectively). Punctate fluorescent spots and aggregates were seen at the cell periphery (arrows) and did not appear to move over time suggesting a possible association with plasmodesmata.

The ORF9:GFP fusion appeared to localize to the nucleus of onion cells (Fig 4j). A potential nuclear targeting signal composed of basic residues, RKKK, is present from positions 123 to 126. Confocal microscopy confirmed the targeting of ORF9 to the nucleus *in planta* (Fig 5g). A human histone 2B protein:GFP fusion (H2B:GFP), which is directly incorporated into the nucleosomes (Kanda *et al*., 1998), was used as a control (Fig 5f). The ORF9 fusion product appeared to be congregating within the nucleolus (Fig 5h). Soluble proteins of M_r less than 50-60K, such as ORF9:GFP, are able to diffuse freely in and out of the nucleolus making it impossible to differentiate with nuclear-targeted GFP fusions (Peters, 1986). It is generally accepted that retention of such a protein in the nucleolus involves an interaction with a nucleolar protein to remain in the nucleolus. This poses an interesting question as to why an protein encoded by an RNA virus would be targeting a host factor in the nucleolus. Given the multifunctional role of the nucleolus several possible activities could be targeted, including host cell cycle regulation (Pederson, 1998), senescence (Olson *et al*., 2000) or interfering with host cell defences such as RNA silencing (Carmo-Fonesca *et al*., 2000). Non-specific RNA binding proteins that diffuse into the nucleus may also become more concentrated in the nucleolus because of the large amounts of rRNA that is present (Hiscox, 2002). The M_r-23K protein encoded by the 3'-terminal gene of CTV has been shown to be an RNA binding protein (Lopez *et al*., 2000) and is the only protein within the family *Closteroviridae* for which RNA binding activity has been demonstrated. The CTV p23 protein has been reported to control asymmetric accumulation of CTV RNAs by downregulating negative-stranded RNA accumulation and indirectly increasing expression of 3' genes (Satyanarayana *et al*., 2002). This protein is similar in size and position in the viral genome to the M_r-24K product of GLRaV-1 ORF9, however no significant sequence homology can be found between them.

The small hydrophobic protein (ORF2) gave the most striking localisation pattern showing spots of unknown identity and of various sizes (Fig 4c). Bioinformatic analysis of the 59 amino acid protein using the PSORT program (Nakai and Horton, 1999) predicted that it is an integral membrane protein targeted to the endoplasmic reticulum (ER) via an uncleavable N-terminal hydrophobic signal sequence (Fig 6a). There was no evidence of ORF2 targeting to the ER in the onion epidermal cell layer. Confocal microscopy of agroinfiltrated tobacco cells, however, gave two distinct patterns of localisation. Initially the ORF2 pattern (Fig 5b) resembled the localisation of the ER targeted control, mgfp5-ER (Fig 5a) (Haseloff *et al*., 1997), with GFP visible on the ER network and in the ER around the nucleus (arrows). The second pattern appeared to be associated with a later stage of ORF2 expression (Fig 5c). The ER pattern was still present, but at a lower level compared to the large aggregate structures which appear to be originating from the ER (arrows).

Multivesicular bodies in ORF2 expressing cells

The ultrastructural changes associated with ORF2 expression were investigated using transmission electron microscopy. mgfp5-ER was used as a control to observe if any changes were caused by the agroinfiltration procedure, GFP expression or processing of plant tissue for TEM. The control cells, repeated in triplicate, consistently showed an organised structure (Fig 7a) compared to the ORF2 expressing cells (Fig 7b). The ER in the ORF2 expressing cells was abnormally forming numerous vesicles with various diameters ranging from 50nm to 150nm (Fig 7c). Large

multivesicular bodies (MVBs) were present consisting of a single membrane filled with vesicles (Fig 7f). These vesicles most likely originated from the ER based on the observed change of the ORF2:GFP localisation pattern from the ER to large cytoplasmic aggregates. The formation of the MVBs probably results from the collection of untargeted vesicles by the late endosome pathway. The cytopathology of all members of the family *Closteroviridae* so far examined is characterised by the presence of vesiculating mitochondria and MVBs localised in phloem cells (Faoro, 1997). The MVBs reported in *Closteroviridae* infections consist of vesicles, 50-100nm in diameter, bound by single membranes and containing viral RNA (Faoro and Carzaniga, 1995). They are believed to originate from the vesiculating mitochondria and are thought to be released into the cytoplasm after the disruption of these organelles. The involvement of the endoplasmic reticulum and tonoplast membranes in the formation of vesicles, hypothesised at first (Faoro *et al*., 1981), has not been confirmed (Faoro *et al*., 1991). These MVBs have been previously referred to as Beet yellows virus-type (BYV-type) vesicles, named after the *Closterovirus* type member (Agranovsky, 1995). Our results indicate that the ORF2 product is the viral encoded protein responsible for the induction of the MVBs originating from the ER. We therefore refer to the ORF2 product as the Vesicle Inducing Protein originating from the Endoplasmic Reticulum (VIP-ER).

How is VIP-ER inducing morphological changes of the endoplasmic reticulum?
PSORT predicted VIP-ER to be a type 1b membrane protein with its N-terminus exposed to the ER lumen, a transmembrane region from residues 14 to 30, and its C-terminus exposed to the cytosol (Fig 6a). A region of hydrophobic moment was found from residues 25 to 40 (Fig 6b), which is predicted to form an amphiphilic α-helix containing a hydrophobic face and a hydrophilic face charged with polar residues. VIP-ER displays characteristics typical of the family of so-called membrane lytic peptides (Segrest *et al*., 1990). Membrane lytic peptides are encoded in numerous genomes of positive-strand RNA animal viruses, such as enteroviruses (Carrasco *et al*., 1995), and have been implicated in the proliferation or reorganisation of cellular membranes and vesicles. This is possibly a mechanism for increasing the available surface area for RNA synthesis. It has been proposed that these cytolytic peptides are able to permeate membranes either by forming multimeric membrane-integral pores or, alternatively, by lying parallel to the lipid bilayer and increasing membrane curvature (Shai, 1995).

Strong similarities can be seen between VIP-ER and the 2B protein of the enterovirus *Coxsackievirus B3.* 2B is also a small hydrophobic protein with the potential to form an amphipathic helix with a structural arrangement typical of lytic peptides (van Kuppeveld *et al*., 1996). The 2B protein has been localised to the rough ER membrane and the outer surface of ER-derived membranous vesicles at which viral RNA replication takes place (Beinz *et al*., 1987, 1994). Early in infection, the 2B protein forms membrane-embedded pores in the endoplasmic reticulum and subsequently disrupts its cation gradient (van Kuppeveld *et al*., 1997). This results in the induction of vesicles from the ER and the increase in plasma membrane permeability facilitating viral replication and release respectively (Kuppeveld *et al*., 1997). The VIP-ER protein of GLRaV-1 is expected to have a similar mode of action. The targeting of VIP-ER to the ER with subsequent formation of ER-derived membrane structures has been observed. If VIP-ER was using the same mode of action as the 2B protein there would be a detectable increase of cytosolic calcium levels. Attempts to observe this expected increase in cytosolic calcium levels using calcium indicators, Indo-1 and Calcium Crimson, have failed due to technical difficulties of introducing the dyes into the plants cells. However, increased cytosolic calcium in ORF2 expressing cells is supported by the observation that mitochondria appear to be swollen size (Fig 7e) relative to those in the control cells (Fig 7d). Mitochondria, along with the ER, play pivotal roles in regulating intracellular Ca^{2+} content. Mitochondria are endowed with multiple Ca^{2+} transport mechanisms by which they take up and release Ca^{2+} across their inner membrane. During cellular Ca^{2+} overload, mitochondria take up cytosolic Ca^{2+}, which in turn induces opening of permeability transition pores (Kowaltowski *et al*., 2001). When the pores open, water and solutes enter the matrix, causing swelling of the mitochondrial matrix.

The role of VIP-ER in the life cycle of GLRaV-1 may not be limited to the formation of multivesicular bodies for the establishment of the viral replication complex and subsequent RNA replication, but may also have an important role in cell-cell movement. This is based on mutagenesis of a full-length construct of BYV, which showed that its small hydrophobic protein, p6, is essential for cell-cell movement (Alzanhova *et al*., 2001). Similar investigations of GLRaV-1 movement proteins cannot occur until a full-length infectious clone is constructed. Viruses generally exploit plasmodesmata, which form a channel spanned by a plasma membrane and contain a thin, appressed tubule of the endoplasmic reticulum, thereby establishing cytoplasmic as well as ER continuity between neighbouring cells (Staehelin, 1997). The association of VIP-ER with the ER during infection and its impact on the morphology of the ER indicates a potential role of the endomembrane system in cell-cell spread of GLRaV-1.

During the preparation of this manuscript Peremyslov *et al*. (2004) reported that the BYV p6 protein is also associated with the rough endoplasmic reticulum, but this protein was unable to induce vesicle formation. Our results indicate that the endoplasmic reticulum-derived vesicle localisation pattern develops over multiple days. This important phenotype was possibly missed in this study since p6:GFP expression was only monitored for two days. Mutagenesis of the BYV p6 protein indicated that there is a very rigid structure-to-function relationship within this protein. The majority of mutations inserted across the protein sequence caused a loss of cell-cell movement. The homodimerisation of the p6 protein via a disulphide bond between N-terminal cysteine residues was also shown to be essential for cell-cell movement. Like VIP-ER some lytic peptides, for example the p10 protein of Avian Reovirus, contain cysteine residues in their cytoplasmic domains, often positioned proximal to their transmembrane domains (Bodelon *et al*., 2002). Mutation of these cysteine residues results in the loss of the ability to induce vesicle formation, but not to associate with membranes. Whether VIP-ER oligomerises with or without the involvement of the cytoplasmic cysteine residues remains to be proven in future studies.

The completion of the genome sequence provides the opportunity to construct a full-length infectious clone of GLRaV-1 and conduct investigations required to further understand the virus-cell interactions that facilitate GLRaV-1 infection. Localisation of the GLRaV-1 proteins within the cell has provided new insight into their function. In particular, the formation of BYV-type vesicles associated with the expression of the VIP-ER protein.

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Figure Legends

Fig. 1. Comparison of the genome organisation of GLRaV-1 with those of other select *Closteroviridae*. Rectangles represent ORFs. Homologous genes are shaded similarly. Open boxes indicate genes with no statistical similarity to other proteins in existing databases. P-Pro, papain-like protease; MTR, methyltransferase; HEL, helicase; POL, polymerase; HSP70, homologue of HSP70 proteins; CP, coat protein; CPd, diverged copy of coat protein.

Fig. 2. Conserved motifs of *Closteroviridae* present in the putative P-Pro and MTR domains of GLRaV-1. The amino acid sequence alignment of GLRaV-1 with the consensus sequences of P-Pro and MTR of BYV, GLRaV-2, CTV, LChV-1, LChV-2, LIYV, PMWaV-2 and GLRaV-3 is shown. The amino acid residues that are identical or similar among the proteins are shown in upper case. The residues conserved between GLRaV-1 and other *Closteroviridae* are shown in bold. The putative catalytic cysteine and histidine residues of the P-Pro domain are marked with an asterisk (*) and an arrow (?) is used to indicate the predicted cleavage site between two glycine residues. The conserved sequence motifs I–IV of the MTR (Rozanov *et al*, 1992) are indicated.

Fig. 3. Phylogenetic analyses of *Closteroviridae* replicases. The viruses are compared based on the similarity between their complete replicase domains, when available, including their P-Pro, MTR, HEL and RdRp domains.

Fig. 4. Transient expression and visualization of GFP fusion proteins in onion epidermal cell layer. Cells were bombarded with expression plasmids encoding the proteins indicated in each panel examined by fluorescence microscopy. Bar=100µm.

Fig 5. Transient expression and visualization of GFP fusion proteins in tobacco epidermal cells. Cells were agroinfiltrated with binary expression plasmids encoding the proteins indicated in each panel examined by confocal laser scanning microscopy. (a-g) Bar=25µm. (h) Bar=100µm.

Fig 6. Bioinformatic analysis of GLRaV-1 VIP-ER protein. (a) Complete amino acid sequence of VIP-ER protein (59-aa) showing the predicted hydrophobic and amphiphilic regions. (b) Hydropathy plot of VIP-ER protein using a window size of nine residues. Positive hydropathy indicates a hydrophobic nature. Negative hydropathy indicates a hydrophilic nature. (c) Helical wheel representation of the putative cationic amphipathic a-helix formed by amino acids 25-40 of VIP-ER. Positively charged residues are circled and hydrophobic residues are boxed.

Fig 7. Transmission electron microscopy of tobacco epidermal cells agroinfiltrated with binary expression plasmids encoding mGFP5-ER (a, d) and VIP-ER (b, c, e and f). (a) Bar=2µm. (b) Bar=1µm. (c) Bar=250nm. (d, e) Bar=500m. (f) Bar=250m.

Figure 1

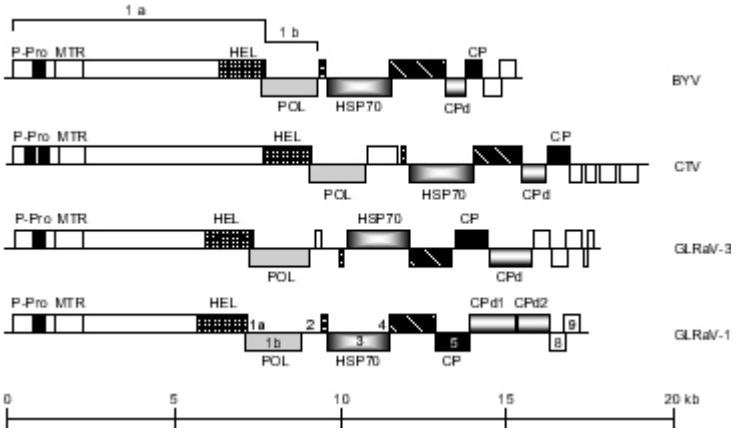


Figure 2



Figure 3

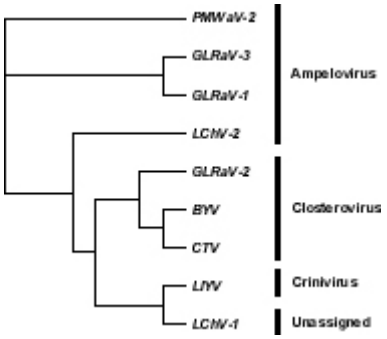


Figure 4

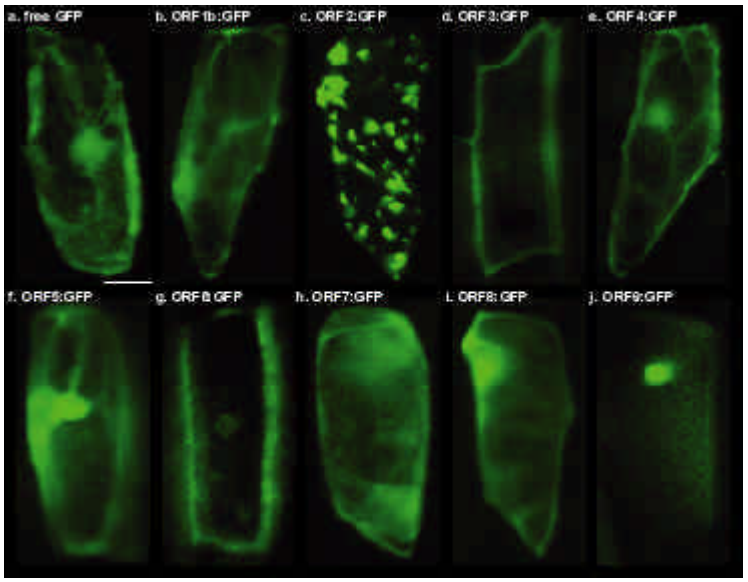


Figure 5

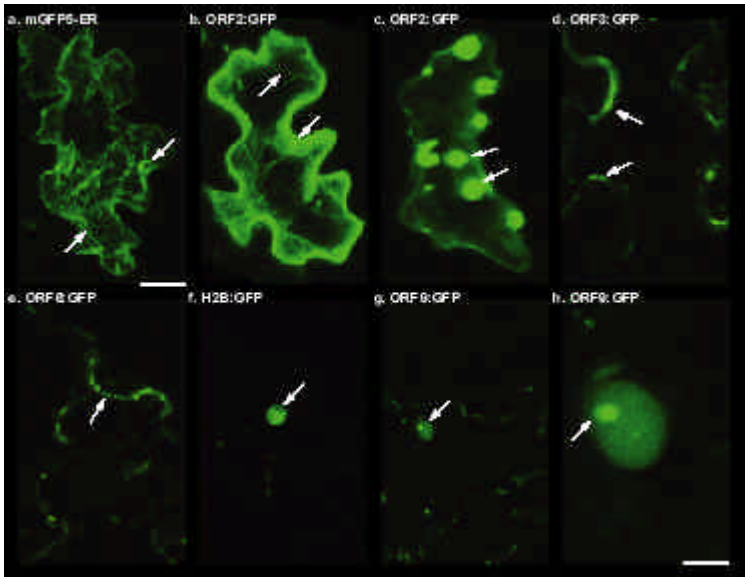


Figure 6

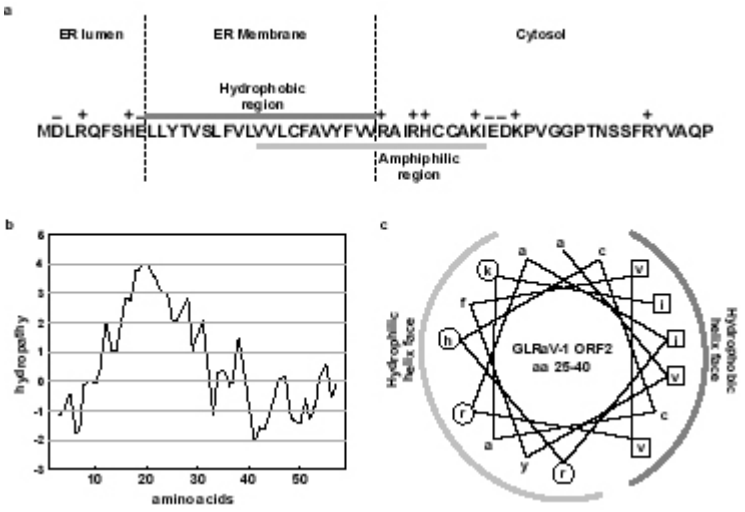
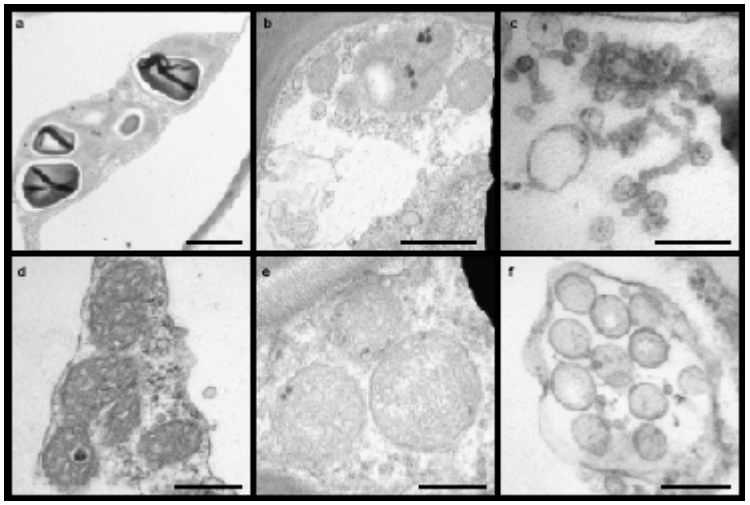


Figure 7


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