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Development of a test for potato leaf roll virus (PLRV) & determination of PLRV strains in South Australia

PT009

Mr JM Simes

Mr RH Symons

The University of Adelaide

Dr CMJ Williams

SA Department of Agriculture

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A METHOD FOR EXTRACTION AND DETECTION OF POTATO LEAFROLL VIRUS (PLRV) BASED ON THE POLYMERASE CHAIN REACTION AND THE SEQUENCE ANALYSIS OF STRAINS OF PLRV PRESENT IN AUSTRALIA.

J.M. Simes[1], C.M.J. Williams[2] and R.H. Symons[1,3] .

1 **Department of Biochemistry, University of Adelaide, ADELAIDE, S.A., 5000.**

2 **Department of Agriculture, Lenswood Horticultural Research Centre, LENSWOOD, S.A. 5240.**

3 **New Address: Department of Plant Science, Waite Agricultural Research Institute, The University of Adelaide, GLEN OSMOND 5064**

INTRODUCTION

Potato Leafroll Virus (PLRV) is a serious pathogen of the potato, causing widespread and devastating crop damage that may result in substantial financial loss. Infection may be termed primary (acquired directly from feeding aphids) or secondary (acquired through the growth of an infected seed source), the latter for which the symptoms are usually of greater severity. The genome of PLRV has been entirely sequenced (Mayo et al., 1989, van der Wilk et al., 1989) and is classified as a member of the Luteovirus family (Harrison, 1984). Luteoviruses characteristically have small isometric capsids containing a single-stranded plus sense RNA genome. They are aphid transmitted in a persistant manner and restricted to the phloem of the host plant (Casper, 1988). These properties make virus propagation difficult and yields of virus are often low. Thus, a method for PLRV detection should avoid the need for virus transfer and be sensitive enough to detect small amounts of virus. Such a detection method would provide invaluble information for crop management, certification of virus-free seed potatoes and assessment of infection in one-year-off-tubers.

Here we report on:

1. A rapid and reliable method for the extraction of nucleic acids from fresh or frozen potato leaves and from cold stored (4°C) tubers.
2. The use of these crude RNA extracts for the detection of PLRV RNA via the Polymerase Chain Reaction (PCR). This technique involves the highly specific amplification of part of the PLRV RNA into double stranded DNA which can be detected by gel electrophoresis.
3. The existence of sequence variations in the coat protein coding region of isolates of PLRV from different regions of Australia.
4. Comparative analysis of our sequence data with published data shows that the coat protein coding region of the genome is highly conserved.

MATERIALS AND METHODS

Full descriptions of the materials and methods used are detailed in Appendix I.

RESULTS

(1) RNA EXTRACTION FROM POTATO TUBERS AND LEAVES

A typical absorbance spectrum obtained from nucleic acid extracts, prepared as described in Appendix I, is shown in figure 1. Values for yield and absorbance 260/230, calculated from spectra, are averaged and compiled in table 1. Leaf tissue yielded approximately 9 fold more crude RNA per gram of tissue than tubers. This is not surprising considering that the tuber is primarily a storage tissue. Leaf and tuber tissue extracts gave an A260/230 of approximately 2.5 and 2.3, respectively, indicative of nucleic acid preparations substantially free of proteins and other UV-absorbing materials.

(2) PCR OF CRUDE RNA EXTRACTS

The PCR is a technique that can enzymatically amplify defined regions of DNA. To apply this to PLRV detection we first created a complementary DNA copy (cDNA) of part of the PLRV RNA genome by reverse transcription using a specific DNA primer 23 nucleotides long (P1, Figure 2), complementary to the PLRV RNA, plus the enzyme reverse transcriptase. Then, using a virus specific primer, P2, designed to bind to a specific region of the PLRV cDNA, we used PCR to amplify the region between the primers. Full experimental details are given in

Appendix I. The result is a product of size defined by the boundaries of the primers, which we can visualise by analytical gel electrophoresis.

Figure 2 simplistically outlines the organisation of the protein coding regions of the PLRV genome, the binding sites for the primer pair used for the PCR and the PCR technique. The primers, P1 and P2, produce a PCR product of 732 base pairs. PLRV could be detected, by PCR, in either tuber or leaf extracts, as shown in figure 3A. A size specific product was produced from PLRV infected leaves or tubers. No band of the same size was produced from healthy leaves or tubers. Figure 3A, lane 6 shows a product but it is not the correct size. A Southern Transfer of these products from the gel to cellulose nitrate paper followed by hybridisation with a nucleic acid probe for the PLRV coat protein gene is shown in figure 3B. The products from PLRV infected samples bound the PLRV probe (dark bands) indicating them to be virus-derived. The band in figure 3A, lane 6, did not bind the probe and is not visible in figure 3B. Therefore, the product is not virus-derived and the sample was not infected. Spurious products of incorrect size were rarely produced.

(3) COMPARISON OF DETECTION OF PLRV BY PCR AND DOT BLOT HYBRIDISATION.

Sensitivity and reliability of PLRV detection by PCR was assessed by comparison with dot blot hybridisation. There was 100 percent correlation between leaf or tuber extracts tested by dot blot hybridisation or PCR (n=40). To assess sensitivity, a selected extract was serially diluted up to 1 in a 10,000 and subjected to both dot blot hybridisation and PCR. The results (figure 4) show that PLRV is detectable by PCR easily at 1/100 dilution and faintly at 1/1000 dilution, whereas using dot blot hybridisations PLRV is only faintly detectable at 1/100 dilution and not beyond this. This indicates that detection of PLRV by PCR is at least 10-fold more sensitive than by dot blot hybridisation.

(4) SEQUENCE ANALYSIS OF PLRV PCR DNA.

Sequence data were obtained from five different PLRV isolates; Lower South East/ Mt. Gambier region, three isolates from the Western Australian Department of Agriculture showing mild, moderate and severe symptoms on *Physalis florianda* and a Mildura capsicum isolate showing severe symptoms of virus infection. Data were collated and analysed using the GCG software package version 6 (Devereux et al., 1984) and compared to the four published PLRV sequences; Australian and Canadian (P.Keese et al., 1990), Scottish (Mayo et al., 1989) and Dutch (van der Wilk et al., 1989). Comparison of nucleotide sequences is shown in figure 5. There are a few single base changes, with a particular clustering in the region between nucleotide numbers 3566 and 3586 (underlined figure 5), which is the 5' untranslated region, and a higher number of changes from nucleotide 3697 to the end.

Figure 6 and 7 show amino acid sequence comparisons for open reading frame (ORF) 4 (coat protein) and ORF 5 (17 k protein), respectively. The ORF 5 is contained entirely within ORF 4 (see figure 2) and codes for a protein of undefined function. Amino acids past position 156 (figure 7) are not true translation products because of the stop translation signals (asterisked). Few changes are observed in either protein. The amino acid changes are summarised in table 2 and their nature (conservative, neutral or radical), indicated. Changes in ORF 4 are all either conservative or neutral and should not dramatically effect the properties of the amino acid at the position of the change. Changes in ORF 5 are less conservative, especially past the stop codon at position 157. These amino acids are not true translation products and not part of the final 17 k protein. The changes observed are indicative of the strict control over the nature of the proteins encoded by both ORF 4 and ORF 5.

DISCUSSION.

The extraction method described in Appendix I produced consistent yields of RNA that could be reliably amplified by PCR. Several other extraction procedures were investigated: omitting the LiCl precipitation, omitting the ethanol precipitation prior to the LiCl step in the method described, the use of chaotrophic extraction buffers such as sodium perchlorate or guanidinium isothiocyanate, or the affinity extraction of RNA with cellulose. However, these methods either did not produce reliable yields of nucleic acid or produced extracts that were inhibitory in the PCR.

The final extraction protocol, as well as producing RNA extracts suitable for PCR, also incorporates several important aspects for viral detection.

- The method allows for appropriate sampling of potato leaves or tubers. One hundred grams, starting weight, of tuber tissue was typically used. By taking a large initial sample of leaves or tubers, the chances of detecting low level infections are increased. This becomes important in primary PLRV infections where only a percentage of the crop will be infected.
- The extraction method is rapid and can essentially be performed in one day, overcoming lengthy and tedious methods for virus preparations.
- The inclusion of the reverse transcription and PCR amplification reactions in the one vessel is a time, reagent and cost saving measure. It is also convenient, halving the number of tubes to be handled.

Thus, the combination of the extraction method with viral RNA detection by PCR lends itself to the rapid and routine analysis of a large number of samples.

The comparison of detection by PCR with detection by dot blot hybridisation shows 100 percent correlation. This suggests that PCR can be used confidently without worry of false negatives or positives due to the experimental test system, although the ever present potential for PCR contamination must be considered and standard procedures to minimise risks employed. Comparisons also show PCR to be at least 10-fold more sensitive than dot blot hybridisation as well as a more rapid procedure.

The use of a different primer pair that would produce a smaller PCR product would probably enhance the sensitivity of detection of PLRV by PCR even further. One of the critical steps in the procedure is the reverse transcription of viral RNA into cDNA prior to PCR amplification. Sometimes the enzyme may be prevented, by RNA secondary structure, from producing a cDNA that is long enough for the subsequent PCR amplification. A shorter product may allow the reverse transcription to become more efficient by producing more cDNA that would be long enough for amplification by PCR. Thus more template is produced for the PCR and therefore the detection system is more sensitive.

We have produced a sequence analysis of the coat protein region of five isolates of PLRV. Few nucleotide changes were seen, although based on these changes the viruses may fall into two groups. The Lower south east and Western Australian severe isolates are similar to the Australian isolate, whereas the Western Australian mild and moderate isolates are more like the Scottish, Dutch and Canadian isolates. However, the data are not sufficient to be definitive about these groupings, although different strains do exist.

The majority of the nucleotide changes are silent (do not affect the amino acid sequence). Generally, the amino acid changes that do occur are conservative or neutral (do not affect the properties of the amino acid at that position). These findings indicate that the coat protein appears to be a highly conserved region of the genome.

The high conservation of amino acids sequence in the coat protein region is not surprising since two proteins are encoded in different frames from the same nucleotide sequence (see figure 5, coat protein ORF 4 and ORF 5). Thus any changes would have to be functionally acceptable to both proteins. This is well illustrated by comparing the amino acid changes in ORF 5 (figure 7) before and after the true stop codon at position 157. Before (in the true coding region) there are few amino acid changes and generally most are conservative. In this region the sequence codes for two proteins. After position 157 there is an increased number of changes of a less conservative nature. In this region the sequence codes only for the coat protein of ORF 4. The corresponding amino acids of ORF 4 show few changes. Thus, before position 157 the changes are silent and conservative in both frames, but after position 157 the changes are less restricted and silent and conserative only in ORF 4. Therefore, the region of the genome encompassing ORF numbers 4 and 5 is highly conserved and may be a good target for a general PCR procedure to detect all PLRV strains.

A comparative full genome analysis between the Australian, Canadian, Dutch and Scottish isolates is documented by Keese et al. (1990). This shows that the coat protein coding region is the most conserved region of the entire PLRV genome.

Comparison of coat protein sequences of three isolates of barley yellow dwarf virus by Vincent et al. (1990) also show this region to be highly conserved in this PLRV related member of the Luteovirus family

CONCLUSIONS.

To summarise, we have developed a method for the detection of PLRV which combines an extraction procedure that maximises leaf or tuber sampling with the polymerase chain reaction, a sensitive method for detection of nucleic acids. The method is rapid and can reliably detect PLRV in crude RNA preparations from field samples of potato tubers or leaves.

The procedure can still be refined and expanded. For convenience and ease of handling of large numbers of samples the procedure could be adapted to an ELISA-like identification system, using multiwell plates and a colorimetric detection assay. This would involve significant further developmental effort.

Our sequence analysis has indicated the presence of different PLRV strains which seem to be either Australian-like or European/Canadian-like. It also demonstrates that the coat protein region of the genome is highly conserved. However, the analysis could be extended to include more isolates and further regions of the PLRV genome. A complete sequence comparison with data from a range of variants could be used to determine the most conserved region of the genome that would be the best target for a general PCR to detect all PLRV strains. It is possible, as indicated by both our work and that of Keese et al. (1990), that this would be the coat protein coding region. A complete strain analysis could also be used to determine the most variable region that would be the best target for a strain specific PCR. Thus, the detection system could also be improved by expanding our knowledge of the strains of virus that may be present.

Finally, although the overall method developed for detecting PLRV could be refined further, it can, at its present stage, be used for the routine screening of leaf and tuber samples. The requirements would be a central, reasonably well equipped, laboratory and a competent laboratory technical officer. It is feasible that such a laboratory could index samples for all potato growing States.

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APPENDIX I.

MATERIALS.

EQUIPMENT

Fast Thermal Sequencer -1 (FTS - 1) from Corbett Research, Sydney was used for heat cycling of PCR reactions.

Oligonucleotide primers

P1: 23 bases long, complementry to PLRV RNA, 5' terminus at nucleotide number 4261.

5' CAG TAG CCG GTT TAT ATT TAG TTT ACC 3'

P2: 27 bases long, homologous to PLRV RNA, 5' terminus at nucleotide number 3530 (numbering system as defined by Keese et al., 1990).

5' GGG TGT TGG TTG TGG GCT TGG AC 3'

ENZYMES.

Avian Myeloblastosis Virus Reverse Transcriptase from Pharmacia LKB Biotechnology, AB, Uppsala, Sweden was used to produce cDNA.

Taq polymerase isolated from *Thermus aquaticus* was purchased from Bresatec Ltd, S.A., and used for the PCR amplification.

Modified T7 DNA polymerase (Sequenase Version 2.0, United States Biochemical Corporation (USB), Cleveland, Ohio) was used for sequencing reactions.

Radionucleotides.

Aqueous alpha-^{32}P-dATP was purchased from Bresatec Ltd., S.A., at a specific activity = 3000 Ci/mmole and concentration of 10 mCi/ml.

Buffers and Solutions.

Extraction Buffer : 10 mM Tris-HCl pH 9.0, 1mM EDTA, 2% (w/v) SDS, 0.2% (v/v) 2-mercaptoethanol.

PCR Buffer : 10 mM Tris-HCl pH 8.3, 50 mM KCl, 375 µM each dNTP, 0.01% (w/v) Gelatin, 0.05% (v/v) Nonidet P40, 0.05% (v/v) Tween 20.

TAE : 80 mM Tris-Acetate, 2 mM EDTA, pH 8.0.

5 x Sequencing Annealing Buffer : 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl, 0.25% Nonidet P40, 0.25% Tween 20

Sequencing Mix 1 : 0.01 M Dithiothreitol, 0.16 µM dGTP, 0.16 µM dCTP, 0.16 µM dTTP, 0.05% Nonidet P40, 0.05% Tween 20, 1.6 mM Tris-HCl pH 7.5, 0.16 mM EDTA, 10 µCi alpha-^{32} P-dATP, 0.6 U Sequenase 2.0.

Sequencing Mix 2 : 0.5 µM dGTP, 0.5 µM dCTP, 0.5 µM dTTP, 50 mM Tris-HCl pH 7.5, 0.5% Nonidet P40, 0.5% Tween 20, 5 µCi alpha-^{32} P-dATP, 2.8 U Taq polymerase.

METHODS.

POTATO TUBER RNA EXTRACTION.

One hundred grams of tissue was collected by boring randomly selected tubers with a knife, sampling approximately 2 g per tuber. The tissue was then blended (Waring Commercial Blendor) with 50 ml of Extraction Buffer. Ten grams of the homogenate was weighed out and retained and the remainder discarded. To the 10 g aliquot a further 5 ml of extraction buffer and 5 ml of phenol (saturated with 0.1 M Tris-HCl pH 9.0) was added. This was mixed, incubated at 65°C for 10', cooled to room temperature and 5 ml of chloroform added. After mixing, the phases were separated by centrifugation at 20,000 x g at room temperature for 10', the aqueous phase removed and re-extracted with phenol/chloroform (1:1) at room temperature. The nucleic acids in the aqueous phase were precipitated on ice for 60' with 0.1 volume of 3 M NaOAc, pH 5.2, and 2.5 volumes of ice cold redistilled ethanol. The precipitate was recovered by centrifugation at 20,000 x g, 0°C, 30' and dried under vacuum. It was then resuspended in 3 ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA (TE pH 7.5). Water (0.1 volume), 0.014 volume 1 M MgOAc (final concentration 0.01M MgOAc) and 0.22 volume of 12 M LiCl (final concentration of 2 M LiCl) were added and the high mol. wt. nucleic acids precipitated on ice for 2 hours. The precipitate was recovered by centrifugation, as above, for 60' and dried under vacuum. The pellet was resuspended in 300 µl TE and ethanol precipitated overnight at -20°C. After centrifugation at 10,000 x g, 4°C for 45' the pellet was washed with 70% (v/v) ethanol, recentrifuged for 15' and dried under vacuum. The pellet was resuspended in 50 - 100 µl of 0.1 mM EDTA and the extract stored at -20°C .

LEAF RNA EXTRACTION

Extracts from either frozen or fresh leaf tissue were prepared in the following manner. The leaves (typically 10 g) were crushed using a roller press (Erich Pollahne, Germany) and the tissue washed off the rollers by the dropwise addition of 2 ml Extraction Buffer per gram of tissue. The slurry was then processed as described for tuber tissue from the 65°C phenol extraction step.

PREPARATION OF ^{32}P-LABELLED DNA PROBES

Probes for PLRV were prepared from a recombinant plasmid pT3Z(f-) containing the coat protein coding region of PLRV, kindly donated by Dr. P. Keese, CSIRO, Division of Plant Industry, Canberra. Plasmid DNA was labelled by nick translation to a specific activity of 0.6 x 10^8 cpm/µg DNA with alpha-^{32}P-dATP. The reactions were performed, following the manufacturers specifications, using a NTK-A kit obtained from Bresatec Ltd., S.A. The DNA was extracted with phenol/chloroform (1:1) in the presence of carrier tRNA, separated from unincorporated label by two ethanol precipitations and dried under vacuum. The pellet was resuspended in 10 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.1% (w/v) SDS, 5 mM 2-mercaptoethanol and stored at -20°C.

DOT BLOT HYBRIDISATION

The procedure was performed as described by McInnes et al. (1989) for the use of nick translated probes.

FIRST STRAND cDNA SYNTHESIS AND PCR AMPLIFICATION.

First strand synthesis of PLRV cDNA and PCR amplification was carried out in a 20 µl reaction in a single vessel. Potato tuber or leaf extracts (0.5 - 1 µl) were denatured by heating at 80°C for 1' then annealed to 0.25 µM (1850 ng/ml) of complementry primer (P1) by slow cooling over 15' to room temperature in 18 µl of PCR Buffer. Reverse Transcriptase (7.5 U) and 0.1 volume of 30 mM $MgCl_2$ were added and the reaction incubated at 42°C for 60'. After cDNA synthesis the homologous primer (P2) was added to a final concentration of 0.25 µM (2250 ng/ml) plus 1.4 U of Taq Polymerase . The reaction was overlayed with paraffin oil and PCR cycled at 94°C, 5', 58°C, 45", 72°C, 1'30" for the first cycle and 94°C, 45", 58°C, 45", 72°C, 1'30" for the subsequent 29 cycles. The reaction mixture (0.1 volume) was electrophoresed for 60' at 80 mamp (1.5% (w/v) agarose, 1 x TAE) and PCR products visualised by ethidium bromide staining.

SOUTHERN TRANSFER

The PCR reaction (0.1 volume) was electrophoresed with ^{32}P-end filled *EcoR1* cut *SPP-1* DNA markers (Bresatec Ltd., S.A.) as described above. The gel was then soaked 2 times for 7' in 0.5 M NaOH, 1 M NaCl, rinsed in glass distilled water and washed twice for 15' in 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl. DNA was transferred from the gel to 0.45 µM nitrocellulose filter paper by capillary transfer overnight (Southern, 1975). The nitrocellulose filter was air dryed and baked in an 80°C vacuum oven for 2 hours. It was then prehybridised, hybridised with the PLRV coat protein nick translated probe, prepared as described above, and washed as described for dot blot hybridisations (McInnes et al., 1989).

LARGE SCALE PREPARATION AND ISOLATION OF PCR DNA.

Large quantities of PCR DNA were synthesised in 2 x 100 ul reactions by scaling up the 20 ul reactions described in this section. The PCR product was purified by native acrylamide gel electrophoresis (2 M Urea, 5% acrylaimide, 1 x TBE). DNA was stained with 0.05% toluidine blue, excised and eluted overnight in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS at 37°C. The supernatant was extracted twice with phenol/chloroform (1:1) followed by two ethanol precipitations. The DNA was resuspended in 0.1 mM EDTA and recovery checked on a 1.5% agarose, 1 x TAE analytical mini gel. Typically, approximately 500-800 ng PCR DNA (732 base pair fragment) was recovered.

DIRECT SEQUENCING OF PCR PRODUCT.

The sequencing protocol used involved:

1. Denaturation of the double stranded PCR DNA and sequencing primer with NaOH.
2. Neutralisation of the reaction with HCl.
3. Annealing, extension and dideoxy termination reactions using Sequenase.
4. Amplification of sequencing products using the PCR. The final amplification step provided a marked improvement in the quality and readability of the sequencing gel. The procedure used was as follows:

One quarter of the PCR DNA recovered above (approximately 0.25 pmol) and 20 pmol of either P1 or P2 in a total volume of 8 ul were denatured with 1 ul of 1 M NaOH at room temperature for 5'. The reaction was neutralised with 1 µl of 1 M HCl and 2 µl of 5 x Sequencing Annealing Buffer and 5 ul of Sequencing Mix 1 added quickly on ice. The tubes were spun and 3.5 µl of the reaction added immediately to 2.5 µl of each of the four dideoxy nucleotide mixes (80 µM each dNTP, 8 µM ddATP, ddGTP, ddCTP or ddTTP in 50 mM NaCl). Reactions were terminated by incubation at 37°C for 3'. The termination reactions were then diluted with 6 µl of Sequencing Mix 2, overlayed with paraffin oil and PCR cycled, 10 cycles (94°C, 45", 50°C, 45", 72°C, 1'). Reactions were stopped by the addition of 8 µl of 95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol, 0.02 M NaOH. The sequence was resolved by electrophoresis through a 0.25 mm, 7 M Urea, 1 x TBE, 6% acrylamide gel. This was fixed (10% acetic acid, 10% ethanol, 10') transferred to Whatman 3MM paper, dried under vacuum for 45' and autoradiographed overnight at room temperature.

TABLE 1. Yields (µg/g tissue) and Absorbance 260/230 values (mean +/- SEM) for potato leaf (n=10) and tuber (n=30) extracts prepared as described in Appendix 1. Values were calculated from A 210 - 320 absorbance spectra (Shimadzu UV - 160).

TISSUE	YIELD	ABSORBANCE 260/230
Tuber	44.60 +/- 2.61	2.30 +/- 0.04
Leaf	394.03 +/- 53.35	2.54 +/- 0.05

TABLE 2. Summary of amino acid changes in ORF 4 and 5 of five Australian PLRV isolates in comparison to the Scottish, Dutch or Canadian isolates of PLRV. Changes are classified by conservation of physico-chemical properties or empirical observations of amino acid substitutions (Dayhoff et al 1972).

ISOLATE	AMINO ACID CHANGE (POS. NO.)	NATURE OF CHANGE
<u>ORF 4</u>		
Severe	Asn - Ser (11)	conservative
Moderate	Val - Met (80)	conservative
Mild	Arg - Pro (48)	neutral
Mt. Gambier	Arg - Pro (48)	neutral
	Ile - Arg (103)	neutral
	Val - Ile (139)	conservative
Capsicum	Ser - Pro (57)	conservative
<u>ORF 5</u>		
Severe	Phe - Tyr (24)	conservative
Mild	Ala - Arg (41)	hydrophilic to basic
Mt. Gambier	Phe - Tyr (24)	conservative
	Ala - Arg (41)	hydrophilic to basic
	Ala - Val (106)	neutral
	Val - Ala (121)	neutral
	Pro - Leu (149)	hydrophilic to aliphatic
	Leu - Pro (152)	aliphatic to hydrophilic
PAST STOP CODON NOT TRUE TRANSLATION PRODUCTS		
	Leu - Arg (166)	conservative
	Ser - Leu (187)	hydrophilic to aliphatic
	Pro - Leu (190)	hydrophilic to aliphatic
	Gly or Arg - Glu (192)	neutral

A

B

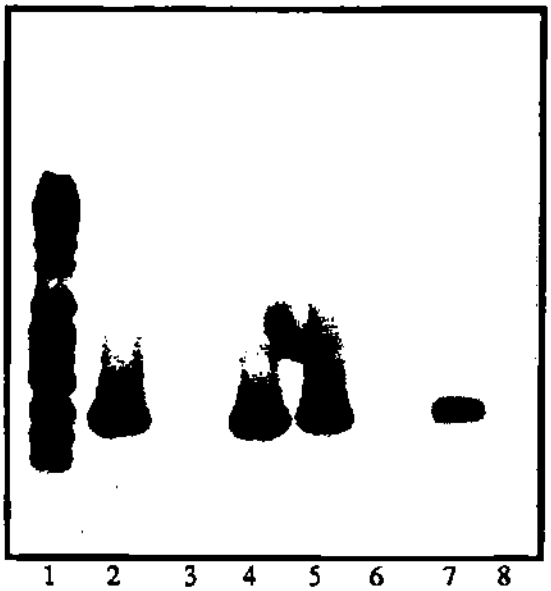


FIGURE 3. Analysis of PCR product. cDNA was made by reverse transcription of viral RNA in potato tuber or leaf extracts and subsequently amplified by PCR as described in Appendix 1. One tenth of the reaction volume was electrophoresed through a 1.5% agarose, 1 x TAE gel. Products were visualised by (A), ethidium bromide staining or (B), Southern Transfer and probing with a ^{32}P nucleic acid probe for the coat protein gene as described in Appendix. Lane 1 = size markers, Lane 2= positive leaf extract, Lane 3 = negative leaf extract, Lane 4 = positive leaf extract, Lane 5 = positive leaf extract, Lane 6 = negative tuber extract, Lane 7 = positive tuber extract, Lane 8 = negative tuber extract.

FIGURE 1. Absorbance ($A_{210-320}$) spectrum for potato tuber nucleic acid extract diluted 3 in 1000 in water. Spectrum determined using a Shimadzu UV-160A UV-visible recording spectophotometer.

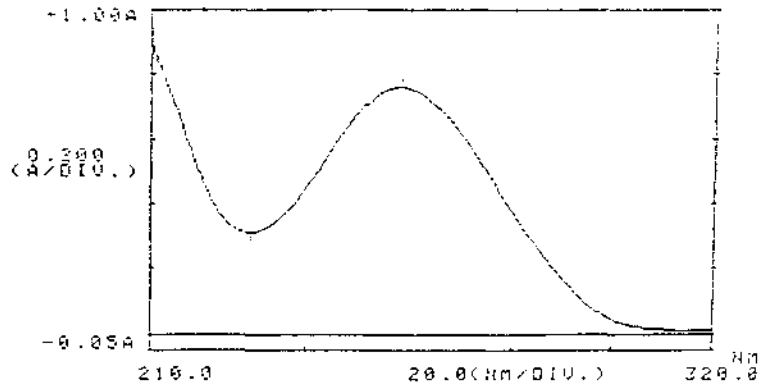
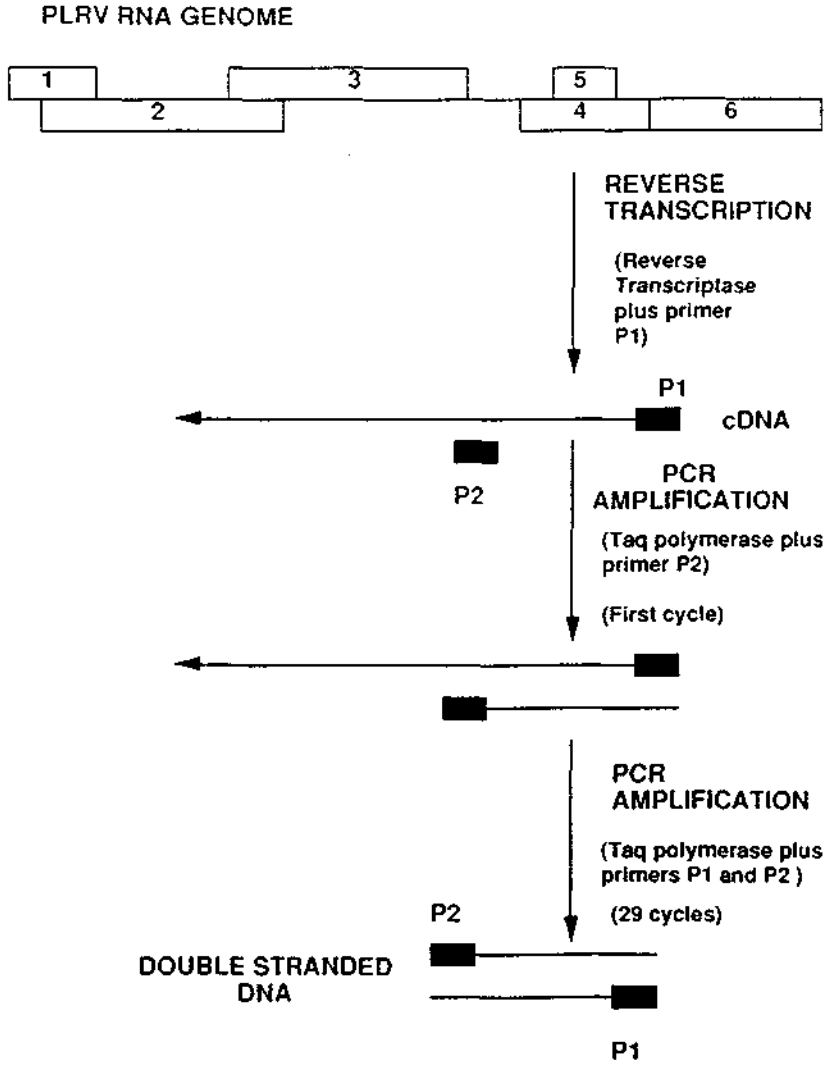


FIGURE 2. Schematic representation of the organisaton of the PLRV RNA genome, binding sites for PCR primers (P1 and P2) and the PCR product. Boxes indicate protein coding regions, defined by numbers; 4 = coat protein coding region. Viral RNA is reversed transcribed into cDNA using P1 and reverse transcriptase. P2 is then added and the PCR is used to amplify the region between P1 and P2 to produce a large amount of product of size defined by the boundaries of the primers. As can be seen, the whole of the coat protein gene is copied into ds DNA.



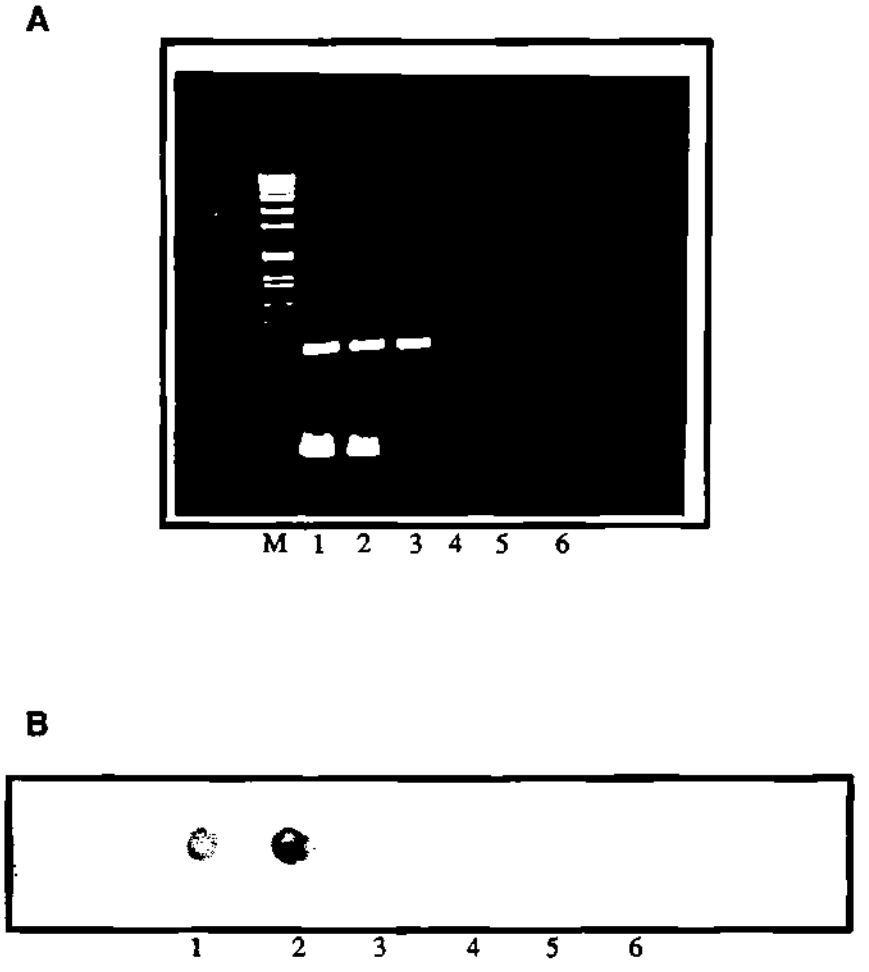


FIGURE 4. Comparison of PLRV detection by PCR and dot blot hybridization. A positive sample was serially diluted then subjected to PCR (A) and dot blot hybridization (B). M = Molecular weight markers, 1 = stock, 2 = 1/10, 3 = 1/100, 4 = 1/1000, 5 = 1/5000, 6 = 1/10,000. All methods used are described in Appendix 1.

FIGURE 5. Comparison of nucleotide sequences of the coat protein region of the PLRV genome from different isolates. Nucleotide changes are highlighted. ?= base not determined. Numbering system is the same as that documented by Keese et al., 1990. ORF 4 = coat protein gene, ORF 5 = 17k protein of undefined function.

```
            3547                                                  3596
CAPSICUM                    ATTTC CGCCCACGTA CGATCAATTG TTAATGAGTA C?????????? ?AAAGGAAAT
WA SEVERE                      T CGACCACGTG CGATCAATCG TTAATGAGTA CGATCGTGGT TAAAGGAAAT
WA MODERATE                 TTTC CTCCCACGTG CGATCAATTG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
WA MILD                     TTTC CTCCCACGTG CGATCAATTG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
MT GAMBIER                    TC CGATCACGTG CGATCTATCG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
SCOTTISH    TTTGTTTACC TAAAGATTTC CTCCCACGTG CGATCAATTG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
DUTCH            TTACC TAAAGATTTC CTCCCACGTG CGATCAATTG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
CANADIAN       TTTACC TAAAGATTTC CTCCCACGTG CGATCAATTG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT
AUSTRALIAN     GTTTACC TAAAGATTTC CGATCACGTT CGATCTATCG TTAATGAGTA CGGTCGTGGT TAAAGGAAAT

            3617                                                  3666
CAP.        GTCAATGGTG GTGTACAACA ACCAAGAAGG AGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTGC
WA SEV.     GTCAGTGGTG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTAC
WA MOD.     GTCAATGGTG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTTC
WA MILD     GTCAATGGTG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTTC
MT GAM.     GTCAATGGTG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTAC
SCOT.       GTCAATGGTG GTGTACAACA ACCAAGAATG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTTC
DUTCH       GTCAATGGCG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTTC
CAN.        GTCAATGGTG GTGTACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTTC
AUST.       GTCAATGGTG GTGCACAACA ACCAAGAAGG CGAAGAAGGC AATCCCTTCG CAGGCGCGCT AACAGAGTAC

            3687                                                  3736
CAP.        AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGC AGAAGAGGAG GCAATCGCCG
WA SEV.     AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGC AGAAGAGGAG GCAATCGCCG
WA MOD.     AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGA??? ????GAGGAG GCAATCG???
WA MILD     AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACCG AGAAGAGGAC GCAATCGCC?
MT GAM.     AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACCG AGAAGAGGAG GCAATCGCCG
SCOT.       AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGC AGAAGAGGAG GCAATCGCCG
DUTCH       AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGC AGAAGAGGAG GCAATCGCCG
CAN.        AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGC AGAAGAGGAG GCAATCGCCG
AUST.       AGCCAGTGGT TATGGTCACG GCCCCTGGGC AACCCAGGCG CCGAAGACGT AGAAGAGGAG GCAACCGCCG

            3757                                                  3806
CAP.        CCCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAA
WA SEV.     CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACA
WA MOD.     ?TCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC
WA MILD     ???AAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCA
MT GAM.     CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC
SCOT.       CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC
DUTCH       CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC
CAN.        CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC
AUST.       CTCAAGAAGA ACTGGAGTTC CCCGAGGACG AGGCTCAAGC GAGACATTCG TGTTTACAAA GGACAACCTC

            3827                                                  3876
WA MOD.     ATGGGCAACT CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
MT GAM.     GTGGGCAACT CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
SCOT.       GTGGGCAACA CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
DUTCH       ATGGGCAACT CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
CAN.        GTGGGCAACT CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
AUST.       GTGGGCAACT CCCAAGGAAG TTTCACCTTC GGGCCGAGTC TATCAGACTG TCCGGCATTC AAGGATGGAA
```

FIGURE 5. CONTINUED.

```
            3897                                                    3946
WA MOD.     TACTCAAGGC CTACCATGAG TATAAGATCA CAAG
MT GAM.     ??CTCA???? ?????????????????????? ???GTATCTT ACTTCAGTTC GTCAGCGAGG CCTCTTCCAC
SCOT.       TACTCAAGGC CTACCATGAG TATAAGATCA CAAGCATCTT ACTTCAGTTC GTCAGCGAGG CCTCTTCCAC
DUTCH       TACTCAAGGC CTACCATGAG TATAAGATCA CAAGCATCTT ACTTCAGTTC GTCAGCGAGG CCTCTTCCAC
CAN.        TACTCAAGGC CTACCATGAG TATAAGATCA CAAGCATCTT ACTTCAGTTC GTCAGCGAGG CCTCTTCCAC
AUST.       TACTCAAGGC CTACCATGAG TATAAGATCA CAAGTATCTT ACTTCAGTTC GTCAGCGAGG CCTCTTCCAC

            3967                                                    4016
MT GAM.     CTCCGCCGGC TCCATCGCTT ATGAGTTGGA CCCCCATTGC AAAATATCAT CCCTCCAGTC CTACGTCAAC
SCOT.       CTCCTCCGGT TCCATCGCTT ATGAGTTGGA CCCCCATTGC AAAGTATCAT CCCTCCAGTC CTACGTCAAC
DUTCH       CTCCTCCGGT TCCATCGCTT ATGAGTTGGA CCCCCATTGC AAAGTATCAT CCCTCCAGTC CTACGTCAAC
CAN.        CTCCTCCGGT TCCATCGCTT ATGAGTTGGA CCCCCATTGC AAAGTATCAT CCCTCCAGTC CTACGTCAAC
AUST.       CTCCGCCGGC TCCATCGCTT ATGAGTTGGA CCCCCATTGC AAAATATCAT CCCTCCAGTC CTACGTCAAC

            4037                                                    4086
MT GAM.     AAGTTCCAAA TTACGAAGGG CGGCGCTAAA ACCTATCAAG CGCGGATGAT AAACGGGGTA GAATGGCACG
SCOT.       AAGTTCCAAA TTACGAAGGG CGGCGCCAAA ACTTATCAAG CGCGGATGAT AAACGGGGTA GAATGGCACG
DUTCH       CAGTTCCAAA TTCCTCAGGG CGGCGCCAAA ACTTATCAAG CGCGGATGAT AAACGGGGTA GAATGGCACG
CAN.        AAGTTCCAAA TTACGAAGGG CGGCGCCAAA ACTTATCAAG CGCGGATGAT AAACGGGGTA GAATGGCACG
AUST.       AAGTTCCAAA TTACGAAGGG CGGCGCTAAA ACCTATCAAG CGCGGATGAT AAACGGGGTA GAATGGCACG

            4107                                                    4156
MT GAM.     ATTCGTCTGA GGATCAGTGC CGGATACTGT GGAAAGGAAA TGGAAAATCT TCAGATCCCG CAGGATCCTT
SCOT.       ATTCTTCTGA GGATCAGTGC CGGATACTGT GGAAGGGAAA TGGAAAATCT TCAGATTCCG CAGGATCCTT
DUTCH       ATTCTTCTGA GGATCAGTGC CGGATACTGT GGAAAGGAAA TGGAAAATCT TCAGATACCG CAGGATCCTT
CAN.        ATTCTTCTGA GGATCAGTGC CGGATACTGT GGAAGGGAAA TGGAAAATCT TCAGATCCCG CAGGATCCTT
AUST.       ATTCGTCAGA GGATCAGTGC CGGATACTGT GGAAAGGAAA TGGAAAATCT TCAGATCCCG CAGGATCCTT

            4177
MT GAM.     TAGAGTCACT ATCAGAGTGG CTTTGCAAAA CCC
SCOT.       CAGAGTCACC ATCAAGGTAG C
DUTCH       CAGAGTCACC ATCAGGGTGG CTTTGCAAAA CCCCAA
CAN.        CAGAGTCACC ATCAGGGTGG CTTTGCAAAA CCCCA
AUST.       TAGAGTCACT ATCAGAGTGG CTTTGCAAAA CCCC
```

FIGURE 6. Comparison of the amino acid sequence of the coat protein (ORF 4) of PLRV isolates. Amino acid changes are highlighted. ?=amino acid not determined. *=stop codon.

```
            1                                                  50
SCOTTISH    MSTVVVKGNV NGGVQQPRMR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRSRRT GVPRGRGSSE
DUTCH       MSTVVVKGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRSRRT GVPRGRGSSE
CANADIAN    MSTVVVKGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRSRRT GVPRGRGSSE
AUSTRALIAN  MSTVVVKGNV NGGAQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRSRRT GVPRGRGSSE
WA SEVERE   MSTIVVKGNV SGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRSRRT GVPRGRGSSE
WA MODERATE MSTVVVKGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRR?? ?GGNR?SRRT GVPRGRGSSE
WA MILD     MSTVVVKGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRPR RG?NRRSRRT GVPRGRGS
MT GAMBIER  MSTVVVKGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRPR RGGNRRSRRT GVPRGRGSSE
CAPSICUM    MST???KGNV NGGVQQPRRR RRQSLRRRAN RVQPVVMVTA PGQPRRRRRR RGGNRRPRRT GVPRGRGSSE

            71                                                 120
SCOT.       TFVFTKDNLV GNTQGSFTFG PSLSDCPAFK DGILKAYHEY KITSILLQFV SEASSTSSGS IAYELDPHCK
DUTCH       TFVFTKDNLM GNSQGSFTFG PSLSDCPAFK DGILKAYHEY KITSILLQFV SEASSTSSGS IAYELDPHCK
CAN.        TFVFTKDNLV GNSQGSFTFG PSLSDCPAFK DGILKAYHEY KITSILLQFV SEASSTSSGS IAYELDPHCK
AUST.       TFVFTKDNLV GNSQGSFTFG PSLSDCPAFK DGILKAYHEY KITSILLQFV SEASSTSAGS IAYELDPHCK
WA SEV.     TFVFT
WA MOD.     TFVFTKDNLM GNSQGSFTFG PSLSDCPAFK DGILKAYHEY KIT
MT GAM.     TFVFTKDNLV GNSQGSFTFG PSLSDCPAFK DGRL?????? ????ILLQFV SEASSTSAGS IAYELDPHCK
CAP.        TFVFTKD

            141                                                190
SCOT.       VSSLQSYVNK FQITKGGAKT YQARMINGVE WHDSSEDQCR ILWKGNGKSS DSAGSFRVTI KVALQN
DUTCH       VSSLQSYVNQ FQIPQGGAKT YQARMINGVE WHDSSEDQCR ILWKGNGKSS DTAGSFRVTI RVALQN
CAN.        VSSLQSYVNK FQITKGGAKT YQARMINGVE WHDSSEDQCR ILWKGNGKSS DPAGSFRVTI RVALQN
AUST.       ISSLQSYVNK FQITKGGAKT YQARMINGVE WHDSSEDQCR ILWKGNGKSS DPAGSFRVTI RVALQN
MT GAM.     ISSLQSYVNK FQITKGGAKT YQARMINGVE WHDSSEDQCR ILWKGNGKSS DPAGSFRVTI RVALQN
```

FIGURE 7. Comparison of the amino acid sequence of the protein encoded by ORF 5 of PLRV isolates. Amino acid changes are highlighted. ?= amino acid not determined. *= stop codon.

```
            1                                                  50
SCOTTISH    MSMVVYNNQE CEEGNPFAGA LTEFSQWLWS RPLGNPGAED AEEEAIAAQE ELEFPEDEAQ ARHSCLQRTT
DUTCH       MSMAVYNNQE GEEGNPFAGA LTEFSQWLWS RPLGNPGAED AEEEAIAAQE ELEFPEDEAQ ARHSCLQRTT
CANADIAN    MSMVVYNNQE GEEGNPFAGA LTEFSQWLWS RPLGNPGAED AEEEAIAAQE ELEFPEDEAQ ARHSCLQRTT
AUSTRALIAN  MSMVVHNNQE GEEGNPFAGA LTEYSQWLWS RPLGNPGAED VEEEATAAQE ELEFPEDEAQ ARHSCLQRTT
WA SEVERE   MSVVVYNNQE GEEGNPFAGA LTEYSQWLWS RPLGNPGAED AEEEAIAAQE ELEFPEDEAQ ARHSCL
WA MODERATE MSMVVYNNQE GEEGNPFAGA LTEFSQWLWS RPLGNPGAE? ???EAI??QE ELEFPEDEAQ ARHSCLQRTT
WA MILD     MSMVVYNNQE GEEGNPFAGA LTEFSQWLWS RPLGNPGAED REEEAIAAQE ELEFPEDEAQ
MT GAM.     MSMVVYNNQE GEEGNPFAGA LTEYSQWLWS RPLGNPGAED REEEAIAAQE ELEFPEDEAQ ARHSCLQRTT
CAPSICUM    MSMVVYNNQE GEEGNPFAGA LTECSQWLWS RPLGNPGAED AEEEAIAAQE ELEFPEDEAQ ARHSCLQRT

            71                                                 120
SCOT.       SWATPKEVSP SGRVYQTVRH SRMEYSRPTM SIRSQASYFS SSARPLPPPP VPSLMSWTPI AKYHPSSPTS
DUTCH       SWATPKEVSP SGRVYQTVRH SRMEYSRPTM SIRSQASYFS SSARPLPPPP VPSLMSWTPI AKYHPSSPTS
CAN.        SWATPKEVSP SGRVYQTVRH SRMEYSRPTM SIRSQASYFS SSARPLPPPP VPSLMSWTPI AKYHPSSPTS
AUST.       SWATPKEVSP SGRVYQTVRH SRMEYSRPTM SIRSQVSYFS SSARPLPPPP APSLMSWTPI AKYHPSSPTS
WA MOD.     SWATPKEVSP SGRVYQTVRH SRMEYSRPTM SIRSQ
MT GAM.     SWATPKEVSP SGRVYQTVRH SRME?S???? ?????VSYFS SSARPLPPPP APSLMSWTPI AKYHPSSPTS

            141                                                190
SCOT.       TSSKLRRAAP KLIKRG**TG *NGTILLRIS AGYCGREMEN LQIPQDPSES PSR*LCKTPN
DUTCH       TSSKFLRAAP KLIKRG**TG *NGTILLRIS AGYCGKEMEN LQIPQDPSES PSGWLCKTPN
CAN.        TSSKLRRAAP KLIKRG**TG *NGTILLRIS AGYCGREMEN LQIPQDPSES PSGWLCKTPN
AUST.       TSSKLRRAAL KPIKRG**TG *NGTIRQRIS AGYCGKEMEN LQIPQDPLES LSEWLCKTPN
MT GAM.     TSSKLRRAAL KPIKRG**TG *NGTIRLRIS AGYCGKEMEN LQIPQDPLES LSEWLCKT
```