PN ACF-001
101394

ANNUAL REPORT

Covering Period [JANUARY 98 - APRIL 99]

Submitted to Human Capacity Development
Bureau for Global Programs, Field Support and Research
U S Agency for International Development

Title of Project

APPLICATION OF MOLECULAR TECHNIQUES TO VIRUS CERTIFICATION IN FRUIT TREES

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Project Number C12-127
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Project Duration March 1995 - January 2000

Executive Summary

The activities during the third year of this project focused on (a) improving and evaluating sensitive detection assays for prunus necrotic ringspot virus (PNRSV) and plum pox virus (PPV) and (b) studying strain differentiation of PNRSV by serological and various molecular methods

PCR-based assays for were further studied in both countries Both immuno-capture (IC)-PCR and ELISA-PCR were evaluated for their sensitivity and reliability for large scale testing in certification programs A comparison with the standard DAS-ELISA assay was included Although both PCR versions were found to be more sensitive than ELISA, the reliability of IC-PCR was limited in several cases, maybe due to inhibitors in the plant sap ELISA-PCR is still being studied Preliminary results show a high sensitivity, however, the cost of reagents may limit its use for large scale testing A PCR assay involving an additional in vitro transcription of the amplified product was applied to PNRSV detection The transcribed products were also used for differentiation among virus isolates (described below)

Differentiation among PNRSV isolates from various hosts and geographic origin was done by bioassays, serotyping with MAbs from two sources, RFLP analysis and transcript conformation polymorphism (TCP), a method based on differences in electrophoretic mobility of RNA transcripts made from PCR products

The serotyping results demonstrated that by using a battery of 4 MAbs it was possible to differentiate all the PNRSV isolates included in this study and determine their serotyping However, the serological variability among virus isolates could not be correlated with symptom expression, host species or geographical origin RFLP results with several endonucleases (EcoRI, AvaII, HincII) differentiated among the isolate groups Similar to the serotyping results the severe isolate (Pl138) was not singled out, however, one other isolate (Apr 152) was different from all the other isolates On the basis of the TCP data obtained with several PNRSV isolates it was concluded that transcription of the PCR products should be made in two orientations 3'- and 5'-end promoters

The PNRSV differentiation results obtained in this project contribute to strain differentiation of PNRSV, however, questions regarding severity and mildness remain open

Section I

A Research Objectives

The specific objectives for the third year of the project are

a Application of target sequence amplification techniques for PNRSV and PPV and development of a sensitive alternative assay for large scale testing of PPV These methods will be compared with conventional ELISA and bioassays
b Study virus strain characterization by biological, serological and molecular methods
c Evaluate fluctuations in virus concentration in infected hosts towards preparation for a 'test calender' for the targeted viruses

B Research accomplishments

The activities during the third year of this project were devoted to

- Development of IC-PCR and PCR-ELISA assays for detection of PNRSV and PPV and comparative experiments with DAS-ELISA
- PCR assays and alternative amplification methods for PNRSV detection and strain differentiation studies
- Biological characterization and variability of PNRSV and PPV isolates
- Serotyping of PNRSV isolates
- Differentiation among PNRSV isolates by RFLP analysis of PCR products
- Sequence analysis of PCR products differentiated by RFLP analysis
- Development and application of alternative molecular methods for PNRSV strain differentiation - Transcript Conformation Polymorphism (TCP) (Israel)

1 Development of IC-PCR assays for detection of PNRSV and PPV and comparative experiments with DAS-ELISA (Hungary)
In the frame of the certification scheme for stone fruit species large number of mother trees has to be tested with a sensitive method All over the world ELISA is used routinely for this purpose although there are certain periods of time when ELISA cannot be used reliably for detection of the decreased virus concentration Different variations of PCR technology seem to be promising for this aim Thus, in this joint project we planned to develop/adapt several PCR variations in order to be able to detect PNRSV and PPV in stone fruit species during the whole year around
In the previous year we reported about the adaptation of RT-PCR technology for PNRSV and PPV and development of one-tube multiplex RT-PCR for detection of mixed infections caused by these two viruses In order to validate these assays in 1998 several trees of different stone fruit species were tested by these methods and compared to the results of ELISA Samples from 45 peach trees, 60 cherry trees and 23 symptom-showing plum trees belonging to different cultivars were analyzed with the conventional RT-PCR and one-tube multiplex RT-PCR for PNRSV and PPV infection ELISA detected 8 PNRSV, 43 PPV and 3 mixed infections Conventional RT-PCR assays revealed 9 PNRSV, 47 PPV-infected samples and 5 mixed infections were found Although in these investigations PCR techniques proved to be more sensitive than ELISA they had also their limitations It was found that the virus was detected by PCR only in 8 out of 23 plum trees with typical symptoms of PPV It

is assumed that the high inhibitor content in plum leaves caused the high number of negative results when the samples were tested by PCR In order to overcome this problem and as RT-PCR assays are very labor intensive in 1998 IC-PCR method was adapted/developed as it does not require complicated RNA prepararation The method developed by Rosner et al (1998) for detection of PNRSV was introduced in the laboratory of Hungary (Krizbai et al , 1998) The same PCR conditions were applied for PPV detection using anti-PPV-IgG from Loewe (Germany) In comparative PNRSV tests 12 peach and 19 cherry trees for the presence of PNRSV, RT-PCR detected 18, IC-PCR 16 infected samples ELISA gave high extinction with 12 samples and a weak reaction with 3 trees In case of testing for PPV of 95 trees of apricots, peaches and plums with unknown health status the following number of infected trees were found RT-PCR 5, IC-PCR 4 and by ELISA 2
In 1999 experiments for modifications of IC-PCR assays are in progress followed by comparative tests with the aim of improving the detectability of PNRSV and PPV in different stone fruit species

2 PCR assays and alternative amplification methods for PNRSV detection and strain differentiation studies (Israel)
The RT-PCR technology for detection of PNRSV in woody hosts, which was developed during the first year of this project and fine-tuned during the second year was put into use both in Israel and in Hungary In addition to RNA-RT-PCR (one tube) reactions, we combined PCR amplification with in vitro transcription of the target sequence by T7 RNA polymerase The T7 polymerase promoter was added to PCR products of PNRSV by using virus-specific primers which also include the promoter sequence This enabled an additional amplification step obtained by in vitro transcription of the PCR products with T7 RNA polymerase Each PCR molecule yielded about a hundred molecules of RNA transcripts In addition to increasing the amount of the amplified virus specific product and reducing non-specific bands obtained by this method, the transcribed products were also be used for PNRSV strain differentiation studies, detailed below

3 PCR-ELISA for detection of PNRSV and PPV (Hungary)
The PCR-ELISA was optimized for sensitive detection of PNRSV and PPV This method is recommended when the virus concentration is very low or the origin of PCR products are unknown (hybridization)
The universal PNRSV capture probe was selected from coat protein gene and labeled with biotin at the 5' end This sequence is present in all known PNRSV isolates with very minor modifications The PPV biotinylated capture probe was developed to the universal 3' non-coding region which is present in all the virus strains (M,D, El Amar and SoC) A 764 bp long cDNA fragment, specific to PNRSV coat protein gene and a 220 bp from 3' non-coding region of PPV was amplified for PCR-ELISA
PNRSV and PPV PCR products were labeled with DIG-dUTP during the amplification and captured by 5' biotinylated probes using streptavidin coated ELISA plate Dig-labeling and colorimetric detection were performed following the PCR-ELISA Kit instructions (Boehringer Mannheim)
PNRSV clone Mission 2116 (plasmid insert) and PPV M strain (maintained on Nicotiana benthamiana) were used in PCR-ELISA in order to check the specificity and sensitivity of this method
Serial dilutions were made with both PNRSV and PPV Dig-labeled PCR products and their detectability was compared on ethidium bromide stained agarose gels and by capturing with

a specific probe (PCR-ELISA) This comparison showed that the sensitivity of the PCR-ELISA was 100X higher than RT-PCR for PNRSV and PPV
Several negative controls were analysed in the same reaction conditions in order to check the cross contamination between samples and the specificity of the probes The specific probes captured only the virus specific PCR products No cross contaminations were detected
Based on results obtained so far it seems that the method using the convenient ELISA format plate is suitable for large scale testing The use of target-specific capture probe allows the differentiation between different isolates, too The method is very sensitive, reliable and relative simple but is more expensive due to the Dig-dUTP and calorimetric detection step Further experiments aimed to evaluate the system using for numerous PNRSV and PPV isolates , are in progress

4 Biological characterization of PNRSV and PPV isolates
The biocharacterization of PPV was continued in Hungary and of PNRSV both in Hungary and Israel
PPV - an experiment was set up to study whether any difference can be found between the PPV M and D strains in the expression and severity of symptom on woody indicators (GF 305, GF 31) and herbaceous species (*C foetidum, Nicotiana benthamiana, N clevelandii)* For this purpose 5 Hungarian PPV isolates based on molecular and serological analyses (nested PCR, RFLP, ELISA using strain specific MABs) were selected The inoculation experiments was extended for 18 new iherbaceous indicator species *Nicotiana debney, N glauca, N suaveolens, N alata, N fruticosa, N rustica, N acuminata, Physalis floridana, P ixocarpa, P pesuviana, Ranunculus acontifolius, R. caleratus, Verbena hastata, V utricifolia, V bonasiensis, Veronica incana, V spicata, V serpyllifolia* Results of the inoculation experiments can be evaluated at the end of 1999
PNRSV - characterization of the 10 selected PNRSV isolates was continued on 10 woody indicator species and 13 additional herbaceous indicator species obtained from abroad in order to involve them to the experiment *Chenopodium album, C amaranticolor, C foliosum, C vulvana, C rubrum , C capoitatum, C botrys, C giganticum, Ammi majus, Origanum vulvare, Ranunculus arvensis, Vigna sinensis* Clones of *C quinoa* were purchased from research institutions and botanical gardens of seven countries
Propagation of these species and the first inoculations were started in 1998 Evaluation of the transmissions under greenhouse conditions and in the field are in progress Among the 7 *C quinoa* clones, the one with large leaves originating from France proved to be the most sensitive This clone was also tested for other viruses and the results were promising Propagation of the French clone is in progress with the aim of gaining large amount of seeds for further experiments
In 1998 characterization of 17 new PNRSV isolates (5 from Israel, 3 from France and 9 from Hungary) on woody and herbaceous indicators mentioned above were also started Results of the inoculation experiments can be evaluated at the end of 1999
In Israel, The biological variability study of isolates of PNRSV collected in Hungary and Israel from infected, locally grown and imported, almond, apricot, peach, plum, a cherry rootstock variety and from rose was completed Isolates originated from traditional cultivars grown commercially for decades in stone fruit orchards (e g Pe25/47, Pl28), selections made locally (e g AlSa, Pma133), imported material during post-quarantine (e g Apr20) and germplasm material (Al63) Data were used for further differentiation studies
Among all the isolates, Pl138 (plum, cv 'Grand Prize') was exceptional in causing

severe stunting and necrotic spots on the source trees maintained in an horticultural evaluation orchard in Hungary since its introduction from California about 40 years ago Symptoms caused by the other isolates on host trees ranged from none to apparent growth reduction and mild leaf symptoms

Graft-inoculations of chip/buds were done onto the woody indicators *P serrulata* cv 'Shirofugen' and GF305 peach seedlings All the inoculated 'Shirofugen' plants reacted with a typical local necrotic reaction around the grafted sample and occasional gum flow Reduced growth was observed with all the isolates on grafted GF305 plants However, only isolate Pl138 caused severe shoot-tip necrosis when graft inoculated on GF305 in the field conditions under cool and relatively humid conditions in west Hungary but very rarely caused these symptoms when assays were performed in milder conditions or in the greenhouse Based on the symptoms observed on natural host trees and supported by graft alysis, we concluded that Pl138 is a severe isolate

5 Serotyping PNRSV isolates (Israel)

Sixteen virus isolates originating from Hungary, Israel and the U S A were included in the serotyping study using MAbs in triple antibody-sandwich TAS-ELISA (Table 2) The assay was performed with dried leaf samples (about 20 mg each) homogenized in extraction buffer (1 75 w/v) PNRSV-specific γ-globulins (AS-0027, DSMZ, Braunschweig, Germany), diluted 1 500, were used for coating plates and trapping virus from diluted plant extracts Two MAbs (NA70C9, N63F10, American Type Culture Collection, Rockville, MD, U S A) and eight MAbs (116, 460, 294, 141, 348, 399, 563, 410) kindly made available to us by Dr Donato Boscia (Universita degli Studi, Bari, Italy) were used as second antibody (details in Table 2) Assay conditions for TAS-ELISA were as previously described by Spiegel *et al*, (1996) DAS-ELISA (with reagents from BIOREBA, Switzerland) was included for comparison

All the tested samples reacted positively with MAb C9 and all but one (Apr152) were positive with MAb 116 Therefore, in addition to PNRSV-specific PAbs, used in a DAS-ELISA assay for comparison, MAbs C9 and 116 were found to have a broad detection range for PNRSV, regardless of the original host or geographic origin, but C9 and 116 did not differentiate among isolates Based on positive and negative reactions of the tested samples with the differentiating MAbs, we divided the isolates into four subgroups (Table 2) MAbs 41, 348, 399 and 563 reacted positively with all five isolates in subgroup 1, and negatively with eleven isolates (except for PlSa) MAbs 294 gave a clear, positive reaction with subgroup 1 (except for AlSa) and six additional isolates (subgoup 2) Apr152 was the only isolate which failed to react with seven differentiating MAbs and 116 and reacted positively only with MAB 460 Therefore, this isolate was considered a separate subgroup (3) Four isolates were included in subgroup 4, of which three failed to react with MAB 294 and two with F10 This subgroup may be tentative, pending accumulation of additional data from a larger number of isolates

The serotyping results demonstrated that by using a battery of MAbs including 116, 460, 294 and either one of the MAbs - 41, 348, 399 or 563 in a TAS-ELISA assay it was possible to differentiate all the PNRSV isolates included in this study and determine their serotyping However, the serological variability among the virus isolates could not be correlated with symptom expression, host species or geographical origin

Table 2 Results of serotyping PNRSV isolates according to reactions with virus-specific monoclonal antibodies

	ELISA[b] Results using MAbs[cd]											
Isolates[a]	C9	F10	116	460	294	141	348	399	563	41	PAbs[e]	Sub-group
AlCha	+	+	+	+	+	+	+	+	+	+	+	
Apr20	+	+	+	+	+	+	+	+	+	+	+	
PlSg	+	+	+	+	+	+	+	+	+	+	+	1
PlOg	+	+	+	+	+	+	+	+	+	+	+	
AlSa	+	+	+	+	-	+	+	+	+	+	+	
Pl138	+	+	+	+	+	+	-	-	-	-	+	
Ro	+	+	+	+	+	-	-	-	-	-	+	
Pe30/4	+	+	+	+	+	nt	nt	nt	nt	nt	+	2
Pm133	+	+	+	+	+	-	-	-	-	-	+	
Pe25/47	+	+	+	+	+	-	-	-	-	-	+	
Pe28/90	+	+	+	+	+	-	-	-	-	-	+	
Apr152	+	-	-	+	-	-	-	-	-	-	+	3
Al63	+	+	+	-	+	-	-	-	-	-	+	
PlSa	+	+	+	-	-	+	+	-	-	-	+	4
Pe67	+	-	+	+	-	-	-	-	-	-	+	
Pl28	+	-	+	+	-	-	-	-	-	-	+	

[a] names and origin of isolates are as in Table 1
[b] TAS- and DAS-ELISA assays performed with MABs and PAbs respectively
[c] MAbs C9 and F10 are abbreviations for NA70C9 and N63F10 (ATCC, USA)
[d] MAbs 116, 460, 294, 141, 348, 399, 563, 41 were from D Boscia, Bari, Italy
[e] PAbs were from Bioreba AG (Reinach, Switzerland)
+ indicates a positive reaction, - a negative reaction and nt = not tested

6 Differentiation among PNRSV isolates by RFLP analysis of PCR products

Based on variability in biological properties and serological data described above, five isolates - AlCha, AlSa, Pl138, Pe30/4 and Apr152, representing subgroups 1, 2 and 3 (Table 2) were initially selected for RFLP analysis, and isolate Ro was later included in the study PCR products obtained with primers pair I/II from selected PNRSV-infected isolates were subjected to RFLP analysis Aliquots (4µl) of the PCR products were digested by addition of EcoRI, AvaII and HincII, followed by an overnight incubation at 37°C The digested products were electrophoresed in a vertical 5% polyacrylamide gel in 40 mM Tris-acetate, pH 8, 1 mM EDTA (TAE) buffer and visualized by staining with ethidium bromide Several other enzymes were also tested – BamHI, KpnI and AluI did not cleave the PCR Products, PvuII cleaved both Pl138 and Apr152 in one identical site and BstXI had a single cleavage site in Pl138 and did not cleave Apr152

Results presented in Fig 1 demonstrate a similar, non-differentiating, cleavage pattern for four isolates - AlCha, AlSa (subgroup 1), Pl138 and Pe30/4 (subgroup 2) with EcoRI, AvaII and HincII A different, unique digestion profile was obtained for Apr152 (subgroup 3) with each of the three tested enzymes (Fig 1A,B,C) We have further compared the restriction pattern of Pl138 (representing the four indistinguishable isolates) with that of Apr152 (subgroup 3) and with an additional isolate originating from rose (Ro) EcoRI cleavage of PCR products obtained from isolates Pl138 and Ro (Fig 2) yielded three fragments of about 285, 150 and 100 bp long indicating the presence of two EcoRI sites A similar cleavage profile was obtained with isolate Pe30/4 (not shown) The PCR products of isolate Apr152 (subgroup 3) were cleaved by EcoRI into two fragments of about 100 and 480 bp indicating the presence of only one cleavage site Based on the EcoRI cleavage patterns, isolates - Pl138, Ro and Pe30/4 (belonging to subgroup 2, Table 2) were indistinguishable from each other, while Apr152 (subgroup 3), could be clearly differentiated from all other isolates Cleavage of Isolates Pl138 and Ro with AvaII resulted in two readily resolvable bands (Fig 2) Two closely migrating fragments - 220 and 205 bp long, were observed in the digest of isolate Ro, in addition to two small fragments of 90 and 40 bp, whereas a single band, of about 210 bp was observed in about the same position in the pattern of isolate Pl138 The AvaII pattern of isolate Pe30/4 was similar to that of Pl138 (not shown) The cleavage pattern of Apr152 differed from the others yielding fragments of 220, 130, 95, 90 and 40 bp, indicating the presence of additional AvaII cleavage sites Among the six tested isolates, only Apr152 was cleaved by HincII yielding two fragments (335 and 240 bp long)

7 Sequence analysis of PCR products differentiated by RFLP analysis

The RFLP results were validated by sequence analysis of the PCR products derived from PNRSV isolates Pl138, Ro, Pe30/4 and Apr152 and mapping the cleavage sites of the various restriction endonucleases PCR products of the virus isolates were cloned into a pGEM-T Easy plasmid vector (Promega, Madison, Wi, USA) according to manufacturer's instructions The standard forward and reverse primers of pUC plasmid were used for sequencing, carried out by the Sequencing Service Center of the Weizmann Institute of Science, Rehovot, Israel The restriction map diagram (Fig 3) demonstrates the similarity among isolates Pl138, Ro and Pe30/4 and the uniqueness of isolate Apr152 with respect to the three tested enzymes – AvaII, EcoRI and HincII (see Figs 1 and 2) Isolate Apr152 lacked an EcoRI site at position 455 (Fig 3) resulting in a larger EcoRI fragment (Fig 2) It has an additional restriction AvaII site positioned at nucleotide no 88 (Fig 3) cleaving the

220 bp fragment into two smaller ones, 125 and 95 bp (Fig 2 and 3) The three isolates, Pl138, Pe30/4 and Apr152, on the other hand, have two identical EcoRI sites and did not have HincII sites corroborating results of the restriction analysis (Fig 2) It seems, therefore, that Apr152 has a genome structure that is markedly different from those of the other isolates studied

8 Development and application of alternative molecular methods for PNRSV strain differentiation - Transcript Conformation Polymorphism (TCP) (Israel)

Differentiation among PNRSV was approached by alternative molecular methods A simple, approach for detection of genomic differences in human cells is the single-stranded conformation polymorphism (SSCP) method developed by Orita *et al*, (1989) This method is based on specific migration rates of denatured single-stranded DNA molecules, conferred by their particular primary and tertiary structures SSCP has been applied for strain differentiation of beet necrotic yellow vein virus, but its sensitivity for detecting mutations was highly variable (Koenig *et al*, 1995) Sarkar *et al*, (1992) hypothesized that SSCP of single-stranded RNA (rSSCP) should have a larger repertoire of secondary structure and proved it to be more sensitive than SSCP in detecting mutations in human factor IX gene

The principle of SSCP was extended by us for differentiation among PNRSV isolates by polymorphism of single-stranded (ss) RNA transcripts copied from amplified virus-specific PCR products In practice, A T7 RNA polymerase promoter was attached to amplified prunus necrotic ringspot virus (PNRSV) sequences by PCR The PCR products than served as a template for transcription Single-stranded transcripts originated from different PNRSV isolates varied in electrophoretic mobility in polyacrylamide gels, presumably because of transcript conformation polymorphism This procedure was applied to differentiate among PNRSV isolates

Four PNRSV isolates RNA (Pl138, Apr152, Ro and Pe30/4), were selected for this differentiation study, based on biological and serological data (Table 1 and 2) Transcripts were made from PCR products of the four PNRSV isolates and their mobilities were analyzed in PAG Transcription of templates with a 3'-end promoter yielded RNAs which exhibited differential electrophoretic mobilities the transcript of isolate Apr152 moved faster than those of the other three (Pl138, Ro and Pe30/4) which all showed the same mobility Transcripts of isolates Pl138 and Pe30/4 made from templates with a 5'-end promoter have the same mobility, while transcripts of isolates Apr152 and Ro were slower RNAs of the four PNRSV isolates transcribed from templates with promoter on the 3' and the 5' ends had about the same mobility, and could not be used for differentiation purposes, whereas the single-strand transcripts could

The PCR product of each virus isolate (which served as a template for transcription) was sequenced, and the degree of nucleotide diversity compared with the sequence of isolate P1138, was determined The highest diversity (about 13%) was found in the sequence of isolate Apr152, less in Ro (~ 4%) and only minor transitions (~ 1%) were spotted in the sequence of the Pe isolate These data were published (Rosner, Maslenin and Spiegel, 1998, enclosed to this report)

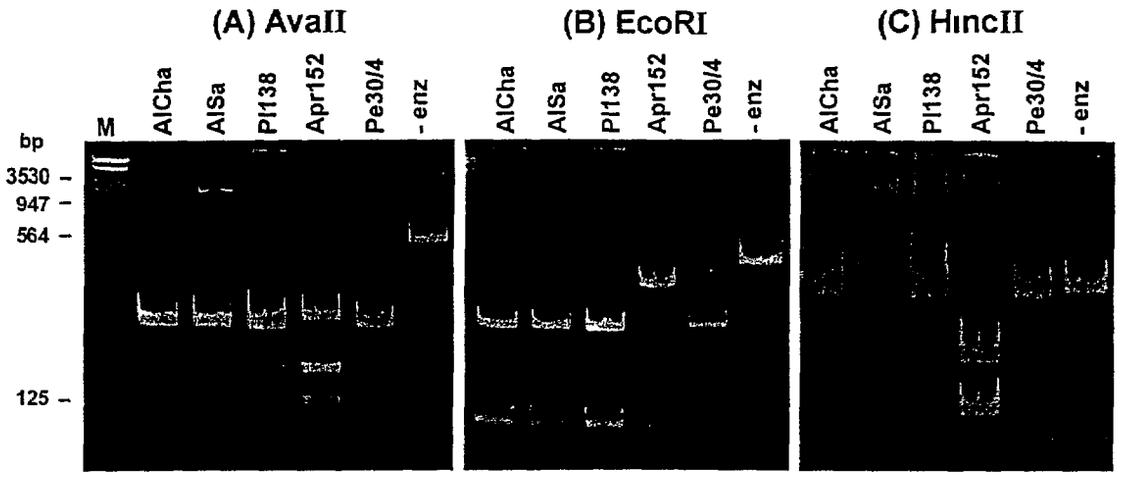

Fig 1 RFLP patterns of PNRSV isolates AlCha, AlSa, Pl138 and Pe30/4 PCR products amplified from total RNA extracted from isolates were cleaved with AvaII (A), EcoRI (B) and HincII (C) Digests were fractionated in 5% PAG and stained with EtBr – enz, uncleaved PCR product of isolate Pl138 M, DNA size marker III (Boehringer, Mannnheim, Germany)

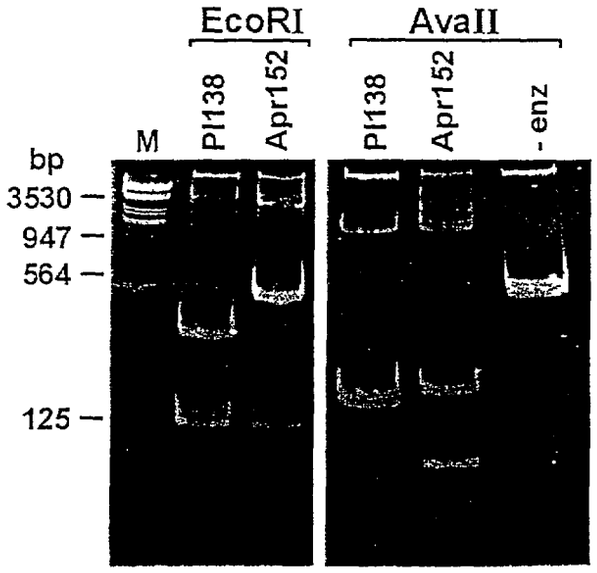

Fig 2 A comparison of cleavage products of PNRSV isolates Pl138, Apr152 and Ro digested with EcoRI and AvaII Details are as in Fig 1

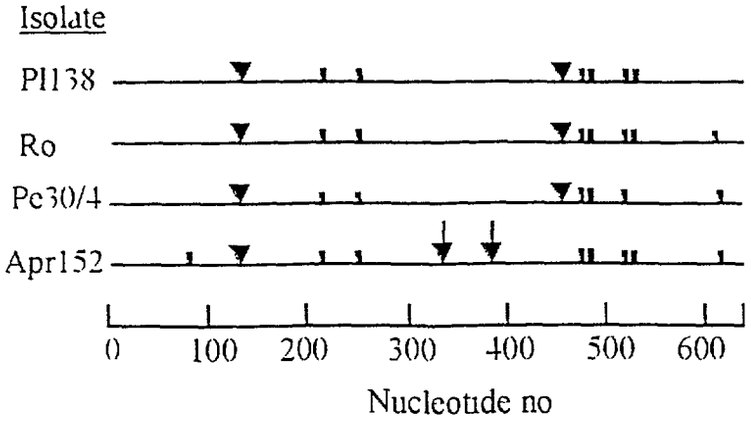

Fig 3 Genomic location of restriction sites of EcoRI, AvaII, and HincII on PCR products (620 bp long, see Materials and Methods) of PNRSV isolates Pl138, Ro, Pe30/4, Apr152
▼ EcoRI, ▮ AvaII, ↓ HincII

C Scientific Impact of Collaboration

Hungary - Dr M Kolber lead the development of large scale NASH method and the ELISA tests Dr M Nemeth lead the biological studies in the field and in the greenhouse Dr G Tokes participated in the ELISA and NASH experiments L Krizbai was responsible for performing the PCR assays

Israel - Dr S Spiegel lead the biological aspects of this project (identification and selection of infected trees, bio-characterization of isolates) and the serological (DAS-ELISA and MABs based assays) differentiation Dr A Rosner lead the development and application of the various PCR techniques and the characterization of PNRSV isolates by molecular methods

D Description of Project Impact

The development of new PCR assays (one-tube and one-tube multiplex RT-PCR) for detection of PNRSV and PPV will provide possibilities to introduce these methods in post-quarantine tests and in certification schemes for production of virus free plant materials Serotyping and molecular characterization studies of PNRSV isolates is resulting in new data added to the current body of knowledge on virus strain differentiation and may lead to better understunding of the disease, caused by this virus It is anticipated that the methods developed in this project will be adopted for detection also for other viruses

E Strengthening of Developing Country Institutions

The establishment and putting into operation of the PCR lab in PHSCS, Budapest, with funding from a USDA OICD grant, contributed to upgrading this diagnostic lab and added a new, much needed, molecular biology dimension to this laboratory
The recent availability of a newly constructed controlled greenhouse (from other funds) in Valence, Hungary, allows in-greenhouse, short-term indexing on woody indicators Obtaining biological data within a short period of time contributes to shortening the testing process and facilitates studying virus isolates It is expected that sensitive methods will be developed for the major fruit tree viruses in Hungary and probably to other viruses too

F. Future work

The activities concerned with this project are generally on schedule with a few exceptions The approval given to our request to end the project in January 2000 (without additional finds) will allow us to continue work on several aspects including season-dependent experiments Based on results (detailed above) that the NASH method was found less sensitive and more time-consuming than the RT-PCR assay we plan to evaluate the recently developed ELISA version of the PCR technology (Rowhani, 1997) for large scale testing

Section II

A Managerial Issues

The activity in this project continued to be intensive in both countries Based on results obtained during the second year several decisions were made In Hungary the NASH method was found less sensitive and more time-consuming than PCR (results detailed above) and not suitable for large-scale testing Therefore, the ELISA-PCR method, published recently, will be evaluated instead In Israel, the 3SR method was unsuccessful, however, several other molecular approaches were sucsessfully developed and applied to selected PNRSV isolates since the second annual report

The extension of the project till January 2000 will allow both groups to complete these tasks and get more season-related data

B Budget

No major changes were made to the budget

C Special Concerns

No protocols which address special concerns were developed

D Collaboration, Travel, Training and Publication

Drs S Spiegel and A Rosner visited the Hungarian laboratory in July, 98 using funds of this project (A Rosner) and other funds (S Spiegel) In addition to a visit to the lab in Budapest, which allowed exchange of information in person and detailed planning of experiments, a field trip including the new facility and discussions with personal on site in Valence was included Dr Spiegel will visit the Hungarian group in June 99 Communication between the Israeli and the Hungarian labs is very frequent by e-mail

Several research papers resulting from this project were compiled and some already published since the second annual report

List of Publications since the second annual report

Krizbai, L , Kolber, M , Nemeth, M , Rosner, A , Spiegel, S 1998
Detection of Prunus necrotic ringspot virus by immunecapture PC (IC-PCR) assay (in Hungarian) Scientific Plant Protection Days'98, Budapest, Abstracts of Papers p 114

Rosner, A , Maslenin, L , Spiegel, S 1998
Differentiation among isolates of prunus necrotic ringspot virus by transcript conformation polymorphism J Virol Methods 74 109-115

Rosner, A , Shiboleth, Y , Spiegel, S , Krizbai, L , Kolber, M, 1998
Evaluating the use of immunocapture and sap-dilution PCR for the detection of prunus necrotic ringspot virus Acta Hortic 472 227-233

Spiegel, S , Tam, Y , Maslenin, L , Kolber, M , Nemeth, M , Rosner, A 1999
Typing prunus necrotic ringspot virus by serology and restriction endonuclease analysis of PCR products (manuscript submitted for publication)

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Journal of Virological Methods 74 (1998) 109–115

Journal of Virological Methods

Differentiation among isolates of prunus necrotic ringspot virus by transcript conformation polymorphism

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Received 5 February 1998 received in revised form 12 May 1998 accepted 12 May 1998

Abstract

A method based on differences in electrophoretic mobility of RNA transcripts made from polymerase chain reaction (PCR) products was used for differentiation among virus isolates A T7 RNA polymerase promoter was attached to amplified prunus necrotic ringspot virus (PNRSV) sequences by PCR The PCR products then served as a template for transcription Single stranded transcripts originated from different PNRSV isolates varied in electrophoretic mobility in polyacrylamide gels presumably because of transcript conformation polymorphism (TCP) This procedure was applied for the differentiation of PNRSV isolates © 1998 Elsevier Science B V All rights reserved

Keywords Prunus necrotic ringspot virus Strain differentiation Ilarviruses PCR Transcription

1 Introduction

Prunus necrotic ringspot ilarvirus (PNRSV) is a positive RNA virus with a tripartite genome which occurs worldwide in *Prunus* spp (Nemeth 1986) Detection of PNRSV in stone-fruit species is carried out during the active growing season by enzyme-linked immunosorbent assay (ELISA) Recently the polymerase chain reaction (PCR) was used for detection of PNRSV in dormant peach trees (Spiegel et al 1996 Rosner et al 1997) PNRSV exists as numerous isolates or strains that vary widely in their pathogenic biophysical and serological properties (Aebig et al 1987 Crosslin et al 1992) Differences in electrophoretic migration of the three genomic RNAs were noted among PNRSV isolates (Crosslin and Mink, 1992) The existence of distinct serotypes may complicate attempts to identify PNRSV infections solely on the basis of serology (Mink et al , 1987)

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PII S0166 0934(98)00076 7

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EVALUATING THE USE OF IMMUNOCAPTURE AND SAP-DILUTION PCR FOR THE DETECTION OF PRUNUS NECROTIC RINGSPOT VIRUS

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Keywords: PNRSV, peach, ilarvirus, PCR

Abstract

Reaction conditions were investigated for the detection of PNRSV in peach trees using immunocapture polymerase chain reaction (IC-PCR) with avian myeloblastosis virus reverse transcriptase (AMV-RT). Incubation of the RT reaction at 46°C resulted in higher levels of amplified products as compared with the recommended 37°C. Preheating the reaction mixture at 55°C for 5 minutes further improved PCR yields. As an alternative to IC, PNRSV could be detected by RT-PCR directly in plant sap. Undiluted sap was negative in PCR, presumably due to the presence of PCR inhibitors. Dilution of sap (at least 1:50) resulted in a successful PCR amplification of the virus-specific DNA fragment. This direct procedure can be easily applied for large scale testing, however, IC-PCR, which also avoids the inhibitors effect, proved to be more sensitive and suitable for low virus titers.

1. Introduction

Prunus necrotic ringspot virus (PNRSV) is an ilarvirus occuring world wide in stone fruits. Virus strains range from highly virulent to symptomless (Fulton, 1985). Reliable detection of PNRSV is required in the fruit tree industry to avoid the costly long term maintenance of virus infected material and biological indexing. Bioassays are time consuming and sometimes inconclusive. The reliability of ELISA is limited due to fluctuations in virus concentration between seasons and uneven distribution in the various parts of infected trees (Scott *et al.*, 1989). Because of these and other problems, alternative molecular techniques for virus detection were applied. The polymerase chain reaction (PCR) method, based on amplification of nucleic acids sequences (both DNA and reverse transcribed RNA) (Doherty *et al.*, 1989), proved to be a highly sensitive diagnostic procedure which is increasingly being used for plant viruses including PNRSV (Spiegel *et al.*, 1996). However, plant tissues vary in the ease with which viral nucleic acids can be purified and in the presence of inhibitory factors. Cell wall composition (e.g. starch), reactive secondary products (e.g. phenolic) and the type of infection (systemic or localized) may have an effect. We have previously demonstrated that the introduction of Sephadex G-50 purification step removed much of these inhibitory substrance(s) from the viral RNA preparation (Vunsh *et al.*, 1991). In the present report we describe another approach using IC and a much simpler sap-dilution protocol to overcome this inhibitory effect.

Reverse transcription of RNA isolated from infected tissue is a crucial step in RT-PCR of RNA viruses. Two RT enzymes - avian myeloblastosis virus (AMV-RT) and molonev murine leukaemia virus (MMLV-RT) (Krug and Berger, 1987) are commonly used in RT-PCR. These enzymes markedly differ in some basic characteristics: the optimal temperature of incubation for AMV-RT is 42°C and it is relatively stable in a diluted solution. The cloned MMLV-RT is active at 37°C and is highly labile. AMV-RT was therefore applied in our research project.

Proc. 17th Int. Symp. on Fruit Tree Virus Diseases
Ed. A. Hadidi
Acta Hort. 472, ISHS 1998

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