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Participating Institutions:

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American University of Beirut, Beirut, Lebanon
Bethlehem University, Bethlehem, Palestinian Authority
Hebrew University, Rehovot, Israel
Ministry of Agriculture, Morocco
National Institute of Health, Bethesda, MD
University of Tunisia, Tunis, Tunisia
University of Wisconsin-Madison

A

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Development of a Regional Viral Indexing and Certification Program for Plant Propagation Materials in the Middle East

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Table of Contents

Executive summary 3

Section I
- Research objectives 4
- Research accomplishments 4
- Scientific impacts of collaboration 10
- Description of project impacts 12
- Strengthening of developing country institutions 12
- Future work 13

Section II
- Management issues 13
- Budget 14
- Special concerns 15
- Collaboration, Travel, Training and Publications 15

Appendix--examples of virus detection protocols 19

Potato virus Y strains, RT-PCR (tech. sheet #1) 20

TYLCV and TYLCSarV, hybridization (tech. sheet #8) 28

PVY strains, RT-PCR, RFLP (tech.sheet #11) 38

PVY, PVX, and PRLV, multiplex RT-PCR (tech. sheet #12) 44

Grapevine virus A, IC-RT-PCR (tech. sheet #22) 48

Grapevine leafroll virus, IC-RT-PCR (tech.sheet #25) 52

Cucurbit yellow stunting disorder virus, RT-PCR (tech. sheet #31) 56

Executive Summary

Nine research laboratories are involved in this project, which includes, Israel, the Palestinian National Authority, Jordan, Lebanon, Egypt, Tunisia, Morocco and USA (two laboratories). Progress towards the research objectives focused on publishing on the WEB the virus detection protocols that have been developed for banana, cucumber, potato, stone fruits, potato, and tomato. Currently, 31 protocols involving molecular and serological approaches are available at the WEB SITE and 10 more are in various stages of preparation:

Homepage: www.plantpath.wisc.edu/InVirLab/docs/merc.htm

Protocols: www.plantpath.wisc.edu/InVirLab/docs/virusindexing.htm

Additional research continued on the development of virus detection methods using protein from a cloned whitefly GroEL gene to capture viruses, and then these capture viruses could be detected by other methods developed in this project. Specific PCR primers are being evaluated for distinguishing the five possible whitefly-transmitted geminiviruses that might occur in tomatoes in this region.

The annual meeting was held in Cyprus in February 2002 and this was the first meeting in which all participants were present. Three students participated in the meeting. A coordinator for each of the four crops, grapevines, potatoes, stone fruits, tomato, was appointed and specific plans for testing viral detection methods were agreed upon by the group. Financial officers from University of Wisconsin-Madison, Hebrew University and Dr. Maxwell visited with each group about their budgets. Most of the group also met in July 2002 in Milwaukee, WI at the meeting of the American Phytopathological Society. Scientists from Israel, Jordan, Morocco and Tunisia worked side-by-side at UW-Madison during the 2002 summer. Students from Palestinian National Authority Additional training involved one student each from Jordan, Lebanon, and PNA to Tunisia, two scientists from Tunisia to UW-Madison, two Moroccan scientists to UW-Madison and one Moroccan scientist to the University of Delaware.

SECTION I

A) Research Objectives:

Because of this grant involves projects, which were added at various times, research funds are currently only available for Jordan, Lebanon, Tunisia and Morocco. The other countries, which include Israel, Egypt, Palestinian National Authority and the two labs in the US, have very limited funds for research.

The original research objectives were as follows:

1. Develop reliable, sensitive detection methods for the major viruses infecting selected propagative materials, i.e., potato, tomato, banana, and stone fruits.
2. Establish a collection of virus antisera and virus detection probes for distribution to private sector and to government institutions in addition to those involved in this project.
3. Establish a Plant Pathogen Detection Lab in the Palestinian Authority.
4. Standardize methods among the virus testing labs by building confidence in the virus detection methods through training and exchange visits and meetings between scientists from the different collaborating countries.
5. Expand collaborative efforts between Arab countries and Israel.
6. Implement a fee-based structure for programs where appropriate.

During this past year, the research teams have focused on the following objectives:

1. Finalizing virus detection protocols for the methods that have been developed or evaluated as part of this project. This would involve maintaining standard viruses.
2. Collaboration between the Arab and Israeli scientists.

B) Research Accomplishments:

Development and evaluation of virus detection protocols: Currently, 31 technical sheets for virus detection protocols have been placed on the WEB (See: www.plantpath.wisc.edu/InVirLab/docs/virusindexing.htm). Each research team has contributed protocols (see examples in Appendix). A standard format was used for all protocols. A list of the virus detection protocols is as follows:

Banana

Banana bunchy top virus

DNA hybridization (radioactive and non-radioactive) #18

Cucumber

Cucumber yellow stunting disorder virus

RT-PCR for *HSP70* gene #31

DNA hybridization (Dig-labeled probe)	#25
Grapevine fanleaf virus	
RT-PCR	#23
Grapevine leaf roll virus	
RT-PCR and IC-RT-PCR	#21
One step, IC-RT-PCR	#25
Potatoes	
Potato virus Y (PVY)	
ELISA	#10
DNA hybridization (radioactive probe)	#6
IC-RT-PCR	#7
RT-PCR (full-length), RFLP	#11
PVY and PVY necrotic strain	
RT-PCR, RLFP-RT-PCR	#1
Potato leaf roll virus (PLRV)	
RT-PCR	#13
RT-PCR-ELISA	#15
Coat protein expression	#17
Potato virus X (PVX)	
RT-PCR-ELISA	#14
PVY, PVX, and PLRV	
Multiplex-RT-PCR	#12
Stone fruits	
Plum pox virus (PPV)	
Coat protein expression	#19
Prune dwarf virus (PDV)	
ELISA	#28
Prunus necrotic ringspot virus (PNRSV)	
Multiplex RT-PCR and RT-PCR-ELISA	#16
Tomatoes	
Tomato yellow leaf curl virus (TYLCV)	
Squash blot PCR	#3
Hybridization (radioactive probe)	#4
Hybridization (non-radioactive probe)	#9
Hybridization (Alk Phosph labeled probe)	#8
Hybridization (Chemi Luminescent method)	#20
ELISA	#2
IC-PCR	#5
RFLP-PCR	#9
TYLCV and *Tomato yellow leaf curl Sardinia virus*	
Dot blot hybridization with Alk Phosph labeled probe	#8

Besides the protocols listed above there are ten that are in various stages of preparation. These include several protocols for detection of viroids of stone fruits and potato, and for detection of different begomoviruses of tomato with specific PCR primers.

One of the major goals of this project is to evaluate virus detection protocols in the laboratories of the various team members. This will be the major focus for next year. For example, the non-radioactive hybridization method with the Alk Phosph labeled probe (#8) was used in the virology class at the University of Delaware this fall and found to be easy to follow by the students.

Using these protocols, research teams have made the following discoveries. In Morocco, the Almeria strain of *Tomato leaf curl Sardinia virus* (TYLCSarV) was detected in mixed infections with TYLCV (Israel type) in tomatoes in two regions of tomato production. In Jordan, TYLCSarV was detected for the first time and it was not known to occur in this part of the Mediterranean region. It was thought to be restricted to the Western Mediterranean region. In Tunisia, mixed infections between TYLCV and TYLCSarV were detected. In these cases, the PCR-amplified fragments were sequenced to confirm the results of the hybridization and PCR analysis. In Palestine, a survey was completed for the grapevine viruses using the protocols developed in other laboratories. In Lebanon, the ELISA methods for PDV and PNRSV were used on field samples. In Tunisia, the protocols listed were used to evaluate the presence of variability in PVY and TYLCV in field samples.

Antisera developed in Thailand and Egypt were evaluated and the antisera from Thailand were very effective in detecting various begomoviruses, whereas the one from Egypt had a very low titer. This will require additional effort to develop this antisera-based kit for TYLCV by the Egyptian team. In general, the research teams have had difficulty expressing coat proteins and developing antisera.

In Israrel, scientists found that a GroEL homologue produced by the endosymbiotic bacteria of *B. tabaci* can be used in the course of a simple test to trap a variety of plant viruses on a solid support and make them amenable to identification by any of PCR, RT-PCR, and ELISA. The test was performed initially using native GroEL partially purified from *B. tabaci*. However, because of the low amount of GroEL that can be prepared from insects, the whitefly GroEL gene was cloned in an expression vector and over-expressed in *E. coli*, ensuring an unlimited supply of the protein. The GroEL produced in bacteria was as efficient in capturing viruses, as was the protein prepared from the insect. In addition to the monopartite TYLCV, GroEL coating PCR tubes was able to capture a number of bipartite begomoviruses from infected leaves such as the whitefly non-transmittable *Abutilon mosaic virus* (AbMV) and the whitefly-transmittable *Watermelon chlorotic stunt virus* (WmCS) and *African cassava mosaic virus* (ACMV). Also, GroEL was able to co-precipitate with two begomoviruses tested, TYLCV and AbMV. GroEL and the virus coat protein were immunodetected in the precipitate. All the begomoviruses tested, TYLCV, AbMV, WmCSV, ACMV, were detectable by ELISA. ELISA plates coated with GroEL were used to trap these viruses. The begomoviruses

were subsequently detected by ELISA using the antibody raised against the TYLCV coat protein.

Besides DNA begomoviruses, these scientists have showed that the recombinant GroEL was able to trap RNA viruses such as the transmitted and non-transmitted strains of *Cucumber Mosaic Virus*. The viruses were identified by RT-PCR using virus-specific primers. CMV was also detected by ELISA-GroEL. These viruses were detected with virus-specific antibodies taken from commercially available detection kits. In our tests, GroEL was unable to trap the potato viruses PVY and PVX and the *Grapevine virus* A.

Since in these assays, the scientists expect GroEL to bind to the virus coat protein, they wondered if it was possible to predict whether or not a given plant virus is able to bind to insect endosymbiotic GroEL. They found that binding virus-GroEL was not related to the shape of the virions since geminate, globular and filamentous viruses do bind GroEL. The viruses able to bind chaperonins have in general a coat protein with a basic isoelectric point, a marked positive charge and are relatively rich in arginine residues. In addition, it seems that these viruses present consensus patterns in the N terminus (between amino acids 27-40) and in the C terminus (between amino acids 197-209) characterized by a sharp shift from a hydrophilic to a hydrophobic region (not shown). Further work is need to confirm, or infirm, these predictions.

The following are examples of the applications of the methods as applied by the Tunisian team:

TYLCV: Analysis of samples by dot blot hybridization under high stringency provide a reliable tool for both identification and quantification of TYLCV. This assays offers a reliable tool to test a large number of samples rapidly. Using degenerate primers, coat protein gene can be amplified from either TYLCV-Is or TYLCSarV genomes. Analysis by PCR-RFLP using *Ava*II allows the assignment of TYLCV isolates into one of the two species: Is isolates produce a restriction pattern of about 370 and 300 bp while that of the Sardinia isolates has three fragments with approximate size of 360, 160 and 150 bp. Our ultimate goal is to develop a simple, rapid and reliable method for diagnosis and typing TYLCV spreading isolates.

PVY: The tissue-blotting assay proved a simple, fast, reliable and very economical method. As previously reported, this simple technique offers numerous advantages when a large number of samples need to be processed rapidly. In this respect, this technique should prove, in the future, very useful for epidemiological studies of PVY. The only key parameter seems to be the quality of the antiserum, which causes interpretation problems, if it gives a significant cross-reaction to healthy plant components. Coat Protein gene amplification by RT-PCR is reliable and very rapid protocol for detection of most of the PVY strains spreading in different hosts: pepper, tomato and potato. RFLP scanning of the whole genome could be seen as a fast highly informative and relatively simple supplement of the established methodologies.

Grapevine viruses: Detection of GLRaV3 in infected grapevine plants was performed. Virus RNA was successfully detected not only in total crude nucleic acid extracts of infected grapevine tissues but also in viruliferous mealybug vector extracts by RT-PCR. This detection was rapid, sensitive and specific without occurrence of any background. A comparative ELISA, RT-PCR and IC-RT-PCR assays were carried out and revealed the greater sensitivity and specificity of PCR techniques. Furthermore, we have optimized the experimental conditions of molecular procedures: IC-RT-PCR and RNA-capture RT-PCR. Theses techniques were efficient to detect GFLV viruses with more sensitivity and specificity than the direct RT-PCR and ELISA methods. A comparative study between IC-RT-PCR and RNA capture RT-PCR showed the efficiency of the nucleic acid capture procedure.

Viroids: In order to develop rapid and specific detection techniques of viroids infecting citrus and grapevine cultures, we have compared biological and molecular protocols. Ideally, these procedures should allow the rapid screening of a large number of samples and some of them should allow the detection of viroids maintained at low levels in the host plant. For nucleic acids extraction from tree leaves, our results showed that phenol protocol is the most laborious and time consuming. The Trizol technique, reported for the first time for viroids purification, is rapid and simple but can be applied only for some plants. Cellulose procedure, avoiding the use of organic solvents, gave a response with all the plants tested when coupled to the RT-PCR. Viroids concentrations as low as 50 pg may be detected by R-PAGE in a routine test. The sensitivity of R-PAGE is similar to that of nucleic acid hybridization. Therefore, R-PAGE makes a suitable methodology for general screening of plant material for the presence of viroids. The polymerase chain reaction is a very powerful method that has greatly facilitated detection of plant pathogens, which would be difficult or time consuming to detect using conventional techniques. Simple and rapid method of nucleic acids extraction in a microfuge tube was developed, which enable the processing of large number samples. These techniques are very suitable for control of a viroid disease, as they can be used to detect infected plants. This is important, since grafting and cutting tools transmit viroids.

The Lebanese team has focused its research on the development and standardization of diagnostic techniques for two stone fruit viruses {*Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV)} and a cucurbit virus, *cucurbit yellow stunting disorder virus* (CYSDV).

Field isolates of PDV and PNRSV were collected and identified by ELISA, using commercially available kits (Sanofi and Agdia). Two sets of primers were designed that will allow the amplification of the full-length coat protein gene of each virus. Restriction sites (*Bam*HI & *Pst*I) were added these primers. Two RT-PCR protocols were developed that will allow efficient amplification of the coat protein of each virus respectively. The coat protein gene was cloned in a PGem vector and transformed into XL1-blue bacterial cells and maintained at –20C. Three clones were obtained for PDV but only one clone was obtained after great difficulty for PNRSV. Sequencing revealed that the inserts effectively corresponded to coat proteins of PDV and PNRSV,

respectively The coat protein gene of PDV was cloned into two separate protein expression vectors: pRSET and pQE 30, pRSET was transformed into BL21 competent cells while pQE was transformed into M15 competent cells. The protein expression using both vector systems was low and did not exceed 150-200 µg protein/ litre while we were expecting in the mg range.

The purified protein gave a strong positive reaction using an ELISA kit for PDV from Agdia. Two rabbits were injected 7 times at weekly interval with the purified protein (native form) at a rate of 100-150 µg/injection using 1:1 mixture with Freunds incomplete adjuvant. One of the rabbits died and antibody collection was attempted from only one rabbit. The crude serum was subjected to three tests: Agar double diffusion, Dot blot Immunoassay (DIBA) and finally ELISA after conjugation of part of the purified antibodies with alkaline phosphatase. Agar double diffusion tests showed that our serum reacted positively with the purified protein (PDV) but not with stone fruit samples infected with PDV. DIBA showed a darker precipitate with samples infected with PDV but sometimes it was difficult to differentiate from healthy controls. To verify this it may be preferred to use chemiluminescence detection rather than colorimetric methods. The ELISA test showed that our titer is very low and the antibody and the conjugate must be diluted 1:50 or even 1:25 to get positive results which are not yet confirmed, because some negative samples gave a false positive reaction.

For PNRSV, it seems that the expressed protein is lethal to the bacterial cells and that is the reason why we had great difficulties in cloning it. If difficulties persist, we intend to clone the truncated gene instead of the whole gene.

Primers were designed for detection of PDV and PNRSV in multiplex RT-PCR. These primers allowed efficient detection of PDV or PNRSV separately or in mixed infection, but in the presence of a single virus and a mixture of four primers in the PCR reaction, the virus is not detected. Modification of the primers, raising the annealing temperature or hot start may be assayed to alleviate this problem.

For CYSDV the same approach was followed, primers were designed for the whole coat protein gene and two restriction sites added: *Sph*I and *Pst*I, following RT-PCR, the coat protein gene was cloned in PGem, sequenced and after verification of the sequence, it was transferred to an expression vector pQE 30. Following transformation into M15 competent cells, protein expression was performed, the level of expression was very low and apparently there is a similar band with lower intensity in the untransformed cells. The trials will be repeated using higher concentration of IPTG.

As the part of the Original MERC grant, Dr. Ahmed Hadidi directed the research at USDA and later NIH and this part of the grant has finished (1998-2001). Dr. Hadidi has submitted the following accomplishments:

"Developed a rapid and sensitive assay for the specific detection of plant viroids using reverse transcription-polymerase chain reaction (RT-PCR)-probe capture hybridization [RT-PCR-enzyme-linked immunosorbent assay (ELISA)]. The assay was applied successfully for the detection of *Potato spindle tuber viroid, Peach latent mosaic*

viroid, or *Apple scar skin viroid* from infected tissue. This system combines the specificity of molecular hybridization, the ease of the colorimetric protocol, and it is at least 100 fold more sensitive than gel electrophoretic analysis in detecting the amplified product. Six to seven hours are needed to complete the RT-PCR-ELISA for viroid detection from infected leaf."

"Demonstrated by molecular, serological, and biological methods that a new virus, termed *Apricot latent virus* (ALV), infects stone fruits and that its coat protein coding region contains *Apple stem pitting virus* (ASPV)-related sequences. ALV coat protein, however differs in size from that of ASPV. Unlike ALV, ASPV infects only pome fruits. Based on sequence data, serological observations, and bioassays ALV has been classified as a new species in the genus *Foveavirus*, typified by ASPV. Subsequently, a RT-PCR assay for direct detection of ALV from stone fruit tissues has been developed."

"Demonstrated that *Apple scar skin viroid* (ASSVd) is associated with a fruit blemishing disease on cultivated pear (*Pyrus communis*) and wild pear (*P. amygdaliformis*) in the Mediterranean region. Moreover, showed that ASSVd, *Pear blister canker viroid* (PBCVd), and *Peach latent mosaic viroid* (PLMVd) naturally infect both cultivated and wild pears. This is the first report which shows that PLMVd and ASSVd naturally infect cultivated and wild pear in the Mediterranean and that PLMVd infects hosts other than *Prunus* spp."

"Developed a PCR-ELISA method for sensitive and rapid detection of the economically important *Faba bean necrotic yellows virus* (FBNYV) from infected plant tissue and viruliferous aphid vector."

"Developed a novel multiplex RT-PCR-ELISA assay for the detection of six viroids in four genera. The assay was applied successfully for the detection and identification of the following six viroid species from infected tissue: *Potato spindle tuber viroid* (*Pospiviroid*), *Peach latent mosaic viroid* (*Pelamoviroid*), *Apple scar skin viroid* (*Apscaviroid*), *Apple dimple fruit viroid* (*Apscaviroid*), *Pear blister canker viroid* (*Apscaviroid*), *Hop stunt viroid* (*Hostuviroid*)."

"Developed standard and multiplex RT-PCR-ELISA assays for the sensitive detection of isolates and strains of the economically important *Plum pox virus* (PPV) and *Prune dwarf virus* (PDV) in stone fruits. The use of a PPV-specific capture probe or a PDV-specific capture probe allowed the detection of each virus in a single RT-PCR-ELISA assay. Simultaneous use of the PPV-specific and the PDV-specific capture probes permitted the sensitive detection of both viruses in a multiplex RT-PCR-ELISA assay. Nucleotide sequence analyses of the cloned RT-PCR- fragments of PPV or PDV obtained from total RNA extracts of infected stone fruit leaves from different geographical locations revealed more than 90% identity with published sequences, which confirmed the identity of each virus isolate or strain investigated."

C) Scientific impact of collaboration (How have collaborating scientists participated in the project during the reporting year?):

One of the main ways in which collaborating scientists interact is at our annual meeting. It was necessary to hold this meeting in a neutral country and it was held in Cyprus in February 2002. This was very convenient for our colleagues form Lebanon, Jordan, PNA, Egypt, and Israel, as the flights were direct, however this was not the case for Tunisia, Morocco and USA. All countries were represented and students/technical support staff or postdoctoral students came from Lebanon, Israel, Egypt, and PNA. At this meeting leaders were chosen, who will have responsibility for organizing activities based on crops. This will continue for 2003-2004 and one of the main task will be to coordinate the evaluation of the virus detection protocols before the next annual meeting in 2004.

Scientists from all countries except PNA were able to attend the National meeting of the American Phytopathological Society in July 2002 in Milwaukee, WI. A one-day meeting of the group for this MERC project and the Tomato Breeding MERC was held in Milwaukee. Also, these scientists spent various lengths of time at UW-Madison ranging from a week to 5 weeks.

Since it was not reasonable to have scientists from Israel and Arab countries exchange visits, it was decided that as many individuals as could arrange their schedules would do research in the UW-Madison laboratory during the summer of 2002. This resulted in the research being done by scientists from Guatemala (CDR project), Morocco, Israel, Jordan, and Tunisia at the same time. Efforts were made to bring a student from Egypt, but after six-months, the student does not have a visa to US. Also, the lead scientists from Lebanon, Israel, and Egypt were able to visit with each other about the various aspects of this project while they were at UW-Madison at the same time.

Additionally, one student from PNA spent 3 months and one student from Jordan spent 1 month in the laboratory at the University of Tunis.

All scientists have communicated via email and at times, it has been necessary to relay all communications via scientists at UW-Madison to the others. Additionally, all research teams have contributed to the virus detection protocols that are on the WEB or will be placed on the WEB.

Also, at UW-Madison this summer was a student from the University of West Indies and one from Germany. These students interacted with the MERC team scientists and were part of projects on virus detection or Marker-Assisted Breeding.

The group at UW-Madison, which includes Drs. M. Nakhla and D. Maxwell as well as Ms. Abby Bettilyon and Michelle Dubis, undergraduate hourly students, have been very busy ordering chemicals, equipment, primers, sequencing clones, transferring emails to colleagues, sending information and materials as requested. Without the hourly students, this would have been impossible to do.

D) Description of project impact:

Each country has used the detection methods they have developed in their research programs. This has resulted in discovery of new viruses, eg. Jordan, Morocco, and Tunisia. The variability within a virus species has been assessed so as to determine, if new viruses are emerging. Host ranges have been studied. These results have contributed to better management of viruses in each country. In Morocco, the detection of two geminiviruses in tomatoes, *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* Almeria strain was communicated to growers and the Ministry of Agriculture (Plant Protection Unit) and Agricultural University of Agadir.

During the coming year, the various protocols will be evaluated and then during the last year (2004), it is expected that this protocols will be transferred the "certification and quarantine laboratories" in each country. Also, workshops will be help in most countries during the next two years to inform local scientists and extension agents of these new virus detection protocols.

E) Strengthening of developing country institutions:

For all the countries recently added to grant (Jordan, Lebanon, Morocco and Tunisia), each country has received training of scientist, and in most cases specific equipment has been added. For example, this project is the first regional project at the Al-Balqa' Applied University and with the aid of this project two labs have been equipped for molecular biology such as, PCR machine, hybridization oven, freezer, micropipettes, electrophoresis units, oven, water bath, and incubator. A PCR machine, gel documentation system, digital camera were purchased for Morocco. New lap-top computers were purchased for Jordan, Morocco, Lebanon, and Tunisia. This greatly aids in presentations at our annual meetings and report preparation. Software for DNA analysis was purchased for Morocco and Tunisia. Scientists from Morocco, Israel, and Tunisia received training in virus detection at UW-Madison. Students from PNA and Jordan received laboratory training at the University of Tunis. Also, one student from Hebrew University was part of the "international research team" at UW-Madison this summer.

In Tunisia as well as other countries, the need for releasing and producing virus and viroid-free plant material either from imported or from local stocks requires the development and improvement of certification programs. Until now, the quarantine and control service of Tunisia assumes certification of such products using biological or serological methods. Such methods are not expensive but time consuming and subject to many problems of sensitivity. Recently, improved knowledge of pathogens genome and the optimization of molecular biology and biotechnology techniques applied to the diagnosis of plant pathogens have stimulated studies in order to develop diagnostic methods that are sensitive, reliable and less time consuming for detecting and thereby, controlling economically important pathogens affecting selected agricultural crops. Yet, the plant pathogen detection team of our laboratory is working on standardization of the developed protocols and transfer them to the quarantine service by acquisition of skills

in their use through training. This will enable Tunisia to stream line its indexing programs and quarantine procedures. Towards this end, the Tunisian project has contributed to the following (second year for Tunisia in this grant):

-Contribution of virus detection methods development for certification and quarantine programs in Tunisia.

-Establishment of a plant pathogen detection laboratory in Tunisia using the virus-indexing methods developed in certification programs. This will support the quarantine service to provide virus-free propagative materials for many crops.

-Protection of vegetables and some stones fruits crops by rapid and reliable testing of imported plant materials for viruses.

-Private sector and government institutions (the Ministry of Agriculture) will be use these new kits and then money will be saved when growers submit samples for testing.

Support of the detection methods by preparation of specific nucleic acid probes for important plant viruses and viroids.

-Provide training to growers and individuals from private sector transfer new technology and standardize diagnostic procedures of government institutions.

-Use as well as possible the official collaboration signed between our laboratory and quarantine service, the Ministry of Agriculture.

The designation of sub-team leaders for each crop is providing opportunities for scientists, other than those at UW-Madison and Hebrew University, to provide leadership for this project.

F) Future work:

The last two years will be devoted to evaluation of the virus detection protocols in at least one other laboratory and standardization of the methods. Then during the last year, staff for the "certification and quarantine labs" in the various countries will be trained and it is expected that some of these protocols will be come the standard for these laboratories. A workshop on *Plum pox virus* is planned for Morocco in March 2003.

SECTION II

A) Managerial issues:

As indicated in the last report, the continued conflict in the Middle East has made it difficult to achieve the scientific exchanges that were original expected. Meeting in neutral countries and having scientists from various countries working in one laboratory at the sametime have been our solutions. Also, it is been noted by some country scientists that they are not allowed to publish with Israeli Institutions on the same research paper. Again, this makes it difficult to demonstrate the achievement of the "MERC goals"; however, regardless of these difficulties, our teams have collaborating on sharing clones, techniques, antisera, etc. They have even worked on the same research problem recognizing that they may not be listed on research publications.

For the institutional constrains, there are several. In Jordan, they have a transportation issue to and from the research center (labs). Their labs are newly constructed and locate in an area, where it is very difficult to reach with normal cars. The University offers only one bus daily for all people working in the lab. The bus reaches the research center between 9:30 and 10:00 A.M. and goes back to Al-Salt between 3:30 and 4:00 P.M. This means that the actual working hours is about 6 hours, which, in most cases, is not enough even to run simple tests or experiments. All our efforts with the University to solve the problem were, unfortunately, unsuccessful. No Internet connection is available for the PI and other investigators in the research center. Communication with other colleagues in other countries is by using private Internet connection. Even at the university center (NOT in the research center which is about 30 km far from the center of the university) the access to the Internet is very restricted and limited. Therefore, in many cases general Internet cafés are used by students and many investigators in the lab to get access to the Internet. Scientific journals and periodicals: due to financial constrains the subscription to scientific journals is very limited. In the central library of the university there are only 4 journals in the field of molecular biology and plant pathology. In case students or investigators require certain article they have (in most cases) to order it directly from the publisher (expensive) or ask colleagues in other countries for help.

At the University of Tunis, there have been problems with computers and disruption of email service. This is being fixed. The conflict between Israeli and Palestine continues and has caused considerable disruption of activities at University of Bethlehem and made it increasing difficult to carry-out the MERC activities. In spite of the continued conflict, two Palestinian students from University of Bethlehem are now (Dec. 2002) Israel at HUJ and Volcani Center receiving training. For three countries (Lebanon, Jordan and Tunisia), it would not be possible to have joint publications with Israeli Institutional scientists as directed by their respective Universities.

A regional workshop on grapevine viruses was planned for the teams from Lebanon, Israel, PNA, and Jordan for fall 2002, but because of the continued conflict in the Middle East, this was cancelled. All partners were invited to participate in a local conference on tomato and grape diseases in Morocco in February 2003 and colleagues from Guatemala, Egypt, PNA, Jordan, and USA will attend. Regional workshops will be planned for 2003-2004, but it will depend on what happens in the Middle East.

B) Budget:

The budget issues have mainly been addressed in the past and mechanisms to overcome these issues have been developed.

There was a major budget revision for Morocco, which has allowed more training for Moroccan scientists and more workshops in Morocco. This was possible, because the indirect charges listed in the original budget were no longer required as a result of restructuring the grant with the Ministry of Agriculture. These budget changes were

approved by USAID Washington in fall 2002. As indicated, the NIH component has finished.

C) Special Concerns (human subjects, intellectual property rights):

None-

D) Collaboration, Travel, Training and Publications:

Collaboration: This was discussed in Section I –C.

Training and Travel:

Mr. Omar Dar-Issa, student, PNA spent 3 months (September to December, 2001) in the Laboratory of Drs. M. Marakchi and Hatem Fakhfakh, University of Tunis, Tunisia. He learned techniques for detection of grape viruses (RT-PCR, IC-RT-PCR, cloning) and production of polyclonal and monoclonal antibodies.

Dr. M. Sedegui, scientists, Morocco spent September 2001 in the laboratory of Drs. Nakhla and Maxwell at the University of Wisconsin-Madison. He had his first exposure to laboratory work in molecular biology on TYLCV. He also spent time at the University of Delaware with Dr. T. Evans learning techniques for the identification of *Plum pox virus*, and he and Dr. Evans will host a workshop on this virus in March 2002.

Ms. Randa Ebeid, research assistant, Jordan spend 3 months (January to April, 2002) in Laboratory of Drs. M. Marakchi and Hatem Fakhfakh, University of Tunis, Tunisia. She received training on the molecular characterization of *Potato virus Y*.

Mr. A. Aziz and Dr. M. Sedegui, scientists, Morocco spend June 2002 in Drs. Nakhla and Maxwell's laboratory at the University of Wisconsin-Madison learning PCR and non-radioactive hybridization methods for detection of TYLCV and TYLCSarV. They also cloned and sequenced PCR fragments.

Dr. Fouad Akad, Hebrew University of Jerusalem, spend August 2002 in the laboratory of Drs. Nakhla and Maxwell at the University of Wisconsin-Madison working at the bench with Drs. H. Fakhfakh and F. Gorsane, University of Tunis, Tunisia on the molecular characterization of begomoviruses from tomato samples collected in Tunisia.

Mr. Mohammad Abhary, graduate student, Jordan started a 7-month training in the laboratory of Drs. Nakhla and Maxwell at the University of Wisconsin-Madison in August 2002. He will be characterizing the begomoviruses associated with tomato samples collected in Jordan as well as construction of an antiviral vector as part of the Tomato-Resistance MERC project.

Ms. Keriayn Smith, graduate student, University of West Indies received training from July to September 2002 on the molecular detection and characterization of begomoviruses from cabbage. Her major professor was part of the Czosnek/Maxwell CDR grant with University of West Indies, Jamaica. She worked closely with the research team from Israel and Tunis.

Ms. Eva Haenssler, Cologne, Germany spent August 2002 in the laboratory of Drs. Maxwell and Nakhla learning basic molecular biology techniques on detection methods for geminiviruses. She worked closely with Ms. Smith and the team from Israel and Tunis.

Drs. Y. Abou Jawdah, Lebanon, Drs. M. Mazyad and A. Shalaby, Egypt, Dr. H. Czosnek spent 2 weeks June/July 2002 in USA visiting the Nakhla-Maxwell Laboratory at the University of Wisconsin-Madison and attending the National meeting of the American Phytopathological Society in Milwaukee, WI. Dr. M. Sedegui, Morocco, Drs. Nakhla and Maxwell, UW-Madison, also attended this meeting.

Dr. M. Sedegui, Morocco, received training in 2002 on *Plum pox virus* detection methods at the University of Delaware with Dr. T. Evans.

All project leaders attended the annual meeting in Cyprus Feb. 2002 as well as Mr. Mohammad Abhary, Jordan, Ms. Hana Sobh and Hala Hourani, Lebanon, and Dr. Faoud Akad, Israel. Ms. Loretta Erdahl, financial person from UW-Madison and Ms. Elinor Slator, financial person from Hebrew University of Jerusalem also attended this meeting and meet with all the different PI's about their budgets. Ms. Abby Bettilyon, undergraduate student from UW-Madison attended the meeting to assist with local arrangements; and since she orders most of the materials, she also helped solve problems with the different project leaders.

Publications:

Abstracts:

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Appendix

Examples of virus detection protocols

Potato virus Y strains, RT-PCR (tech. sheet #1) 20

TYLCV and TYLCSarV, hybridization (tech. sheet #8) 28

PVY strains, RT-PCR, RFLP (tech.sheet #11) 38

PVY, PVX, and PRLV, multiplex RT-PCR (tech. sheet #12) 44

Grapevine virus A, IC-RT-PCR (tech. sheet #22) 48

Grapevine leafroll virus, IC-RT-PCR (tech.sheet #25) 52

Cucurbit yellow stunting disorder virus, RT-PCR (tech. sheet #31) 56

TECHNICAL SHEET No. 1

VIRUS DETECTION: *Potato virus Y* (PVY) and PVY^N

METHOD: RT-PCR

General

Virus detected: PVY from potato tubers and leaf.
General method is reverse transcription PCR (RT-PCR).

Developed by

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Date: Oct. 8, 2002

Goals

Sensitive method for PVY detection based on RT-PCR.

Introduction

PVY strains are pest quarantine in Israel, and therefore development of sensitive methods for their detection is pivotal for establishing virus free potato seeds. Israel imports all potato seeds for planting from Europe, where the PVY^N strain is present. The current method routinely used for PVY detection is ELISA.

PVY is a member of the *Potyviridae*, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm). Currently, three main strains of PVY are know. (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is designed the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^O. (iii) PVY^N was recognized in the 1950's as causing serve veinal necrosis in tobacco. I was originally described from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^C strains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. There is, currently, consider effort to better

understand the relationship between the different strains of PVY, there genomes and biological properties.

Materials and Methods

Design of primers

The figure below shows the partial sequence of p1 protease gene of PVY-Hungarian N-strain (M95491) and the location of the primers used to discriminate between PVY strains:

```
1      AAATTAAAAC AACTCAATAC AACATAAGAA AATCAACGCA AAAACACTCA CAAAAGCTTT
       TTTAATTTTG TTGAGTTATG TTGTATTCTT TTAGTTGCGT TTTTGTGAGT GTTTTCGAAA
                                                     primer 2
61      CAACTCTAAT TCAAACAATT TGTTAAGTTT CAATTTCGAT CTTCATCAAA CAAACTCTTT
        GTTGAGATTA AGTTTGTTAA ACAATTCAAA GTTAAAGCTA GAAGTAGTTT GTTTGAGAAA

121    CAATTTCAGT GTAAGCTATC GTAATTCAGT AAGTTATTTC AAACTCTCGT AAATTGCAGA
       GTTAAAGTCA CATTCGATAG CATTAAGTCA TTCAATAAAG TTTGAGAGCA TTTAACGTCT

181    AGATCATCCA TGGCAACTTA CACATCAACA ATCCAGATTG GTTCCATTGA ATGCAAACTT
       TCTAGTAGGT ACCGTTGAAT GTGTAGTTGT TAGGTCTAAC CAAGGTAACT TACGTTTGAA

241    CCATACTCAC CCGCTCCTTT TGGGCTAGTT GCGGGGAAAC GAGAAGTTTC AACCACCACT
       GGTATGAGTG GGCGAGGAAA ACCCGATCAA CGCCCCTTTG CTCTTCAAAG TTGGTGGTGA

301    GACCCCTTCG CAAGTTTGGA GATGCAGCTT AGTGCGCGAT TACGACGGCA AGAGTTTGCA
       CTGGGGAAGC GTTCAAACCT CTACGTCGAA TCACGCGCTA ATGCTGCCGT TCTCAAACGT


primer 3
361    ACTATTCGAA CATCCAAGAA TGGTACTTGC ATGTATCGAT ACAAGACTGA TGCCCAGATT
       TGATAAGCTT GTAGGTTCTT ACCATGAACG TACATAGCTA TGTTCTGACT ACGGGTCTAA

421    GCGCGCATTC AAAAGAAGCG CGAGGAGAGA GAAAGAGAGG AATATAATTT CCAAATGGCT
       CGCGCGTAAG TTTTCTTCGC GCTCCTCTCT CTTTCTCTCC TTATATTAAA GGTTTACCGA

481    GCGTCAAGTG TTGTGTCGAA GATCACTATT GCTGGTGGAG AGCCACCTTC AAAACTTGAA
       CGCAGTTCAC AACACAGCTT CTAGTGATAA CGACCACCTC TCGGTGGAAG TTTTGAACTT

541    TCACAAGTGC GGAAGGGTGT TATCCACACA ACTCCAAGGA TGCGCACAGC AAAAACATAT
       AGTGTTCACG CCTTCCCACA ATAGGTGTGT TGAGGTTCCT ACGCGTGTCG TTTTTGTATA

601    CGCACGCCAA AATTGACAGA GGGACAAATG AACCACCTTA TCAAGCAGGT GAAGCAAATT
       GCGTGCGGTT TTAACTGTCT CCCTGTTTAC TTGGTGGAAT AGTTCGTCCA CTTCGTTTAA

661    ATGTCAACCA AAGGAGGGTC TGTTCAACTG ATTAGCAAGA AAAGTACCCA TGTTCACTAT
       TACAGTTGGT TTCCTCCCAG ACAAGTTGAC TAATCGTTCT TTTCATGGGT ACAAGTGATA

721    AAAGAAGTTT TGGGATCACA TCGCGCAGTC GTTTGCACTG CACACATGAG AGGTTTACGA
       TTTCTTCAAA ACCCTAGTGT AGCGCGTCAG CAAACGTGAC GTGTGTACTC TCCAAATGCT

781    AAGAGAGTGG ACTTTCGGTG TGATAAATGG ACCGTTGTGC GCCTACAGCA TCTCGCCAGG
       TTCTCTCACC TGAAAGCCAC ACTATTTACC TGGCAACACG CGGATGTCGT AGAGCGGTCC

841    ACGGACAAGT GGACTAACCA AGTTCGTGCT ACTGATCTAC GCAAGGGCGA TAGTGGAGTT
       TGCCTGTTCA CCTGATTGGT TCAAGCACGA TGACTAGATG CGTTCCCGCT ATCACCTCAA
                                   primer 1
901    ATATTGAGTA ATACTAATCT CAAAGGACAC TTTGGAAGAA GCTCGGAGGG CCTATTCATA
       TATAACTCAT TATGATTAGA GTTTCCTGTG AAACCTTCTT CGAGCCTCCC GGATAAGTAT
```

In order to detect PVY^N, we carried out a sequence comparison between PVY strains. The sequences were retrieved from GenBank and compared. Multiple alignments did

not show many differences between the strains. Minor differences were found in restriction enzyme site (such as an *Hin*dII site) that allowed us to use the polymorphism PCR method (or RFLP-PCR).

RFLP-PCR is based on the different DNA fragment patterns obtained, after the PCR products are incubated with restriction enzymes and separated by agarose gel electrophoresis. These polymorphisms were characteristic of the different PVY strains sequences.

Three primers were designed to amplify PVY strains and to allow the detection of the *Hin*dII polymorphic site of PVY^N. The location of the primers on the sequence of PVY is indicated above.

Primer 1, which is a complementary sense primer (identical in all strains) was used for first strand cDNA synthesis (from viral RNA purified. 5'TTCCAAAGTGTCCTTTGAG3'

Primer 2, which is a sense primer (identical in all the strains), was used to amplify the first cDNA strand. 5'CTTCATCAAA CAAACTCTTT3'

Primer 3, which is a second complementary sense primer, is located between primer 1 and primer 2. The sequence of primer 3 is specific to PVY^N strains and is different from all other PVY groups (PVY^C strains, PVY^O). 5'ATCTGGGCATCAGTCTTG3'

RNA purification from potato tubers and from leaves using the Tri-REAGENT method

1. Homogenize 0.2 g of plant tissues with 750 µl Tri-reagent (Molecular Research Center, Inc.); incubate for 5 min at room temperature.
2. Add 200µl chloroform, shake the sample vigorously for 15 sec and incubate the mixture for 15 min at ambient temperature.
3. Centrifuge at 14,000 rpm for 15 min and collect the aqueous phase to a new tube.
4. Add 0.5 volume of isopropanol and incubate at –20 °C for 30 min.
5. Centrifuge at 14,000 rpm for 10 min, discard the supernatant.
6. Wash the pellet with 70% ethanol and dry under vacuum.
7. Dissolve the pellet with 20 µl ddH_2O, incubate at 70°C for 15 min, store at –80 °C.

cRNA synthesize

1. To 5 µl RNA extract add 8 µl ddH_2O and 1 µl primer 1(100 pmoles); incubate at 70 °C for 10 min, then incubate in ice bath for 30 min.
2. Add 1 µl of each dNTPs (25 mM each), 4 µl reverse transcriptase 5 x buffer, 1 µl AMV reverse transcriptase (Promega); incubate at 42 °C for 1h.
3. Heat for 10 min at 90 °C; adjust the volume to 50 µl with ddH_2O

PCR

1. The PCR reaction contains 5 µl from the reverse transcriptase reaction, 0.25 µl 25 mM dNTPs, 1 µl each primers 1, 2 and 3 (100 pmololes each), 2.5 µl Taq polymerase 10 x buffer and 1 unit *Taq* DNA polymerase; add ddH_2O to a final volume of 25 µl.

2. Cycle: 1 cycle of 95°C for 3 min 50°C for 2 min, 72°C for 2 min; then 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; additional cycle of 72°C for 10 min.
3. Subject the reaction products to 1% agarose gel electrophoresis.

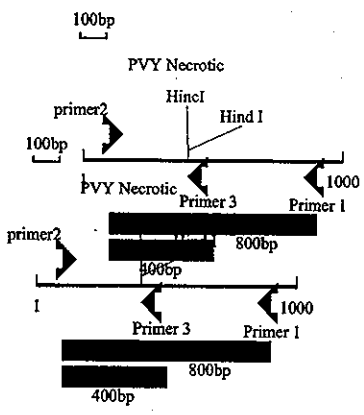
One step RT-PCR

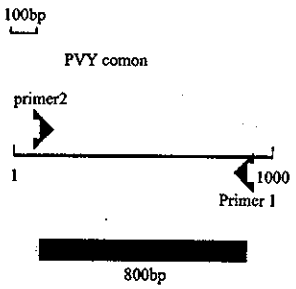
We are now carrying out the RT-PCR reaction in one step using the Access RT-PCR system (Promega). The advantage of this method is that the reverse transcription and the PCR steps are all done in one tube, cutting down manipulations, time and price.

1. To 5 μg total RNA disolved in 10μl ddH_2O and 1μl from each one of the three primers (final concentration 10 μM).
2. Transfer the solution to PCR tubes, heat at 70 °C for 15 min; cool on ice for an additional 10 min.
3. Add the reaction mix, which contain 5μl AMV/T*FI* reaction buffer x5, 2 μl 25 mM $MgSO_4$, 2μl dNTP mix (10mM each dNTP), 0.5μl T*FI*-DNA polymerase (5U/μl), 0.1μl AMV reverse transcriptase (5U/μl); adjust the volume to 25 μl with ddH_2O.
4. PCR conditions: first strand synthesis: 1 cycle at 48 °C for 1h followed by one cycle 94 °C for 3 min. Second strand cDNA synthesis and PCR amplification: 35 cycles at 94 °C for 45 sec, 58 °C for 1 min and 68 °C for 2 min.

Discrimination between PVY strains using RT followed by PCR with three primers

In the case of PVY^N infection, PCR amplification results in the production of two DNA fragments (one migrates at ~ 800bp and the other at ~ 400bp). In contrast, only one DNA fragment amplified (~ 800bp) with PVY^C infected material.



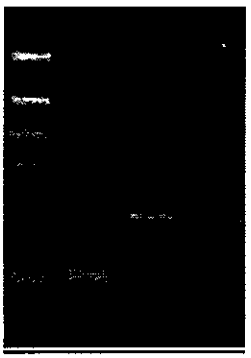


Results

RT-PCR discrimination between PVY strains

In the case of PVY^N infection, PCR amplification results in the production of two DNA fragments (one migrates at ~ 800 bp and the other at ~ 400 bp). In contrast, only one DNA fragment amplified (~ 800 bp) with PVY^C infected material.

M PVY^N PVY^C ?



M: molecular weight marker.
PVY and $PVY^{C:}$ leaves infected with the relevant viruses.
?: unknown sample.

Discrimination between PVY strains by RFLP-RT-PCR

The RT PCR product were cut with *Hin*dII and subjected to PCR using the three primers. In the case of PVY^N, only the P2-P3 400 bp will appear. In the case of PVY^C, only the P2-P1 800 bp will appear.

The figure below shows the pattern obtained with PVY^N and PVY^C.

M PVY^C PVY^N

Discrimination between PVY strains by RFLP-RT-PCR

The RT PCR product were cut with *Hin*dII and subjected to PCR using the three primers. In the case of PVY^N, only the P2-P3 400 bp will appear. In the case of PVY^C, only the P2-P1 800 bp will appear.
The figure below shows the pattern obtained with PVY^N and PVY^C.

M PVY^{C} PVY^{N}

Discussion

The strain PVY^{N} does not exist in Israel, therefore we cloned this fragment from infected plant samples supplied as positive control with the ELISA detection kit (Bioreba). This allowed us to have a positive control for our tests and for preparing PVY-specific probes. Following RT-PCR with the primers 1 and 2, the 800 bp amplified fragment was cut out from the gel, cleaned and cloned into the pGEM^{R} –T Easy plasmid, using the pGEM^{R} –T Easy system protocol (Promega).

A unique restriction site (*Hin*dII site) present only in the PYV^{N} PCR fragment allows discrimination between virus strains.

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methods for differentiating North American PVYNTN. J. Virol. Methods 103:145-156.

Confirmed by:

Edited, Oct. 28, 2002 by D. P. Maxwell

TECHNICAL SHEET No. 8

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV) species using non-radioactive technique for dot blot hybridization

Developed by:

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Date: November 2, 2002

Goals:

Detect and distinguish between TYLCV species by a non-radioactive hybridization.

Advantages:

- Can be used in many laboratories, since it is a non-radioactive technique.
- Quick and reliable method.

Introduction

Tomato-infecting whitefly transmitted geminiviruses (begomoviruses) are a complex of strains and species. Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998): 1) from the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991), 2) from Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSarV) (Kheyr-Pour et al., 1991), 3) from tropical Africa (Senegal, Tanzania), 4) from Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is

PREVIOUS PAGE BLANK

evidence of recombination (Chatchawankanphanich and Maxwell, 2002; Navas-Castillo et al., 2000).

Information about geminiviruses can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae). Also, other web sites are:

http://gemini.biosci.arizona.edu/

http://life.bio2.edu/ICTVdB/29030043.htm

http://www.im.ac.cn/vide/descr840.htm

Materials and Methods

Summary of different steps in this technique:

1. Tissue samples are collected and stored in manner so that they don't decompose (E.G., cut young leaves in very small pieces (1 mm strips) and then let dry for at least 48 hours on filter paper).

2. Membrane spotting: Sample is ground in a buffer and debris removed by an appropriate method (see Dellaporta/heat technique). Small amount (5 μl) is spotted on neutral nylon membrane, and then dried for about half hour.

3. Lysing: prior to hybridization, membranes are treated with denaturing solution to form ssDNA, which binds to the nylon membrane and will be available for hybridization with a nucleic acid probe.

4. Fixing: UV-cross linker can be used to cross link the DNA to the membrane.

5. Preparing the probe and adding it to the membrane: This is the hybridization step. The membrane and labeled probe are placed in a hybridization solution. The hybridization conditions can varied so that the DNA probe will hybridize with the DNA of the test organisms when the percent DNA similarity is 65-75 % (low stringency) or greater than 90% (high stringency). Low stringency would be used to detect any begomovirus in the sample and high stringency conditions would be used if difference geminiviral species were being detected.

6. Washing: membranes are washed in a mild salt/detergent solution to remove any probe that was not hybridized to the test DNA.

7. The film is exposed for hours to days.

DNA Extraction

(J. Potter et al., 2003)

1. Extract DNA from sample in extraction buffer: Plant tissue to be tested are: TYLCV positive control, unknown sample, tissue from non-infected tomato.
2. Obtain a small amount of tissue (2 cork borer for fresh or frozen tissue, 5 mg for dry tissue) and place in a microfuge tube.
3. Resuspend sample in 500 µl of Dellaporata buffer.
4. For dry tissue, leave on ice for 20 min with 500 µl Dellaporta.
5. Grind using a Kontes pestle.
6. Incubate at 65^0 C for 5-10 min.
7. Spin for 10 min at 14,000 rpm.
8. Remove supernatant to new tube. Discard tissue debris.
9. Dilute 1:10 and 1:100 for PCR. Use without dilution for hybridization.
10. Store at –20 ^{0}C.

Dellaporta Buffer: total of 50 ml

- 150 µl BME (beta mercaptoethanol) Fisher BP 176-100
- 5 ml 1 M Tris pH 8.0 Fisher BP 152-5
- 5 ml 0.5 EDTA pH 8.0 Fisher BP 120-500
- 5 ml 5 M NaCl Fisher S 271-10
- 34 ml dds H2O

Membrane Lysing

1. In a large glass tray, saturate 3 layers of Whatman (Cat# 3030917) 3 mm paper with 0.5 N NaOH. (20 ml 5M NaOH +180 ml H_2O). Remove any excess.
2. Lay membranes DNA side up on saturated paper, create no bubbles or folds. Leave for 5 min (saturate paper again before lysing another set of membranes).
3. Transfer membrane to tray with 1 M Tris pH 7.4, leave for 5 min agitating.
4. Transfer membranes to a tray with 2X SSC (20 ml 20X SSC + 180 ml H_2O) (20X SSC: dissolve 175.3 g of NaCl + 88.2 g sodium citrate in 800 ml H_2O, adjust pH to 7.0 with 10 N NaOH. Adjust the volume to 1 liter with H_2O, autoclave).
5. Leave for 5 min agitating occasionally.
6. Transfer membranes to tray with 95% ethanol. Leave for 5 min, agitating occasionally. Ethanol will get cloudy, replace if lysing many membranes.
7. Air dry membranes (1/2 hour).

DNA Fixation on Membrane

There are two methods:

1. UV cross-link DNA by selecting optional cross-link on machine or by exposing to UV light with trans-illumination device for 3 min.
2. Bind the DNA to the nylon membrane by baking for 2 h at 80 ^{0}C.

Preparation of DNA probes

One of the easiest ways to prepare a DNA probe is to use the viral amplified fragment from a PCR reaction with appropriate primers and target DNA.

General Probe: For a general probe (about 500 bp) for Eastern Hemisphere begomoviruses, sequences of several viruses were aligned and the most conserved region of the coat protein (*CP*) gene determined. Primers were designed that would amplify this highly conserved region, which corresponds to the 3' end of the *CP* gene, from TYLCV (Israel) isolate (Navot et al., 1991):

PTYCPv (PTYv369) (viral sense) 5'- ACGCCCGCTCGAAGGTTCG-3'

PTYCPc (PTYc1023) (comp. sense) 5'-CGTCAGCCATATACAATAACAAGGC-3'

A full-length, infectious clone (pEG1) of the TYLCV from Egypt was generally used as the target DNA.

TYLCV (Israel)-specific probe: The viral species-specific probe was the intergenic region (common region, ori region), which is the most variable among different begomovirus species.

Two different sets of primer pairs could be used for TYLCV (Israel) (Navot et al., 1991):

One primer set was designed by Navas-Castillo et al. (1999):

MA-30 (viral sense): 5' GAGCACTTAGGATATGTGAGG 3' (anneals at nt 2566-2586)
MA-31(comp. sense): 5' AGTGGATCCCACATATTGC 3' (anneals at nt 152-170)

One primer set was designed by M. K. Nakhla (University of Wisconsin-Madison:

PTYv21 (viral sense): 5' GTTGAAATGAATCGGTGTCCC 3'
PTYc287 (comp. sense): 5' TTGCAAAGACAAAAAACTTGGGACC 3'
(Number in primer name corresponds to nt position in genome.)

TYLCSarV-specific probe: The primer set was designed by Navas-Castillo et al. (1999) for the Sardinian species of TYLCV, TYLCSardiniaV (Kheyr-Pour et al., 1991):

MA15: 5'-GGATCCCACATATTG-3'
MA14: 5'-TGCATTTATTTGAAAACG-3'

PCR conditions: The thermocycler used was Thermocycle PTC-0200 DNA Engine (Alpha unit). Program: (94 C, 45 sec, 55 C,1:00 min, 72 C, 1:45 min) x 30 cycles, followed by (94 C, 1:00min, 55 C, 1:00 min, 72 C, 5:00min) x 1 cycle, then 4 C.

PCR reaction mixture: *Taq* DNA polymerase in storage buffer B; Catalog: M1661, Promega:

H_2O	12.9 µl
Buffer (Taq) 10X	2.5 µl
Mg++ (Taq) 10 X	2.5 µl
Primer(+) 10 µM	1.0 µl
Primer (-) 10 µM	1.0 µl
dNTP's 10X	2.5 µl (each dNTP at 2.5 mM)
Taq DNA polymerase	0.1 µl
DNA sample	2.5 µl
Total:	25 µl

Target DNA could be either DNA extracted from infected plants as describe above or plasmid DNA with an appropriate insert. Other DNA extraction methods outlined in Rojas et al. (1993) could be used.

Cleaning the DNA

DNA precipitation from PCR reaction:

Combine volume from two 50-µl PCR reactions, measure the volume and adjust to 400 µl. Add 0.1 volume of 3 M sodium acetate, (about 40 µl in 400 µl), add 2.5 volume 100% ETOH (about 1 ml), vortex and incubate at – 80 C for 30 min or –20 C for 4 h, spin at 14K for 15 min, keep pellet and add 70% ETOH spin at 14K remove ETOH, dry in speed vac, resuspend in H_2O 50 µl, shake for 5 min, run gel to see how much DNA you have.

Considerations:

- The concentration of salt in the probe DNA to be labeled should be as low as possible not exceed 50 mM. This is why you do the precipitation step above.
- The DNA concentration should be accurately determined and adjusted to 10 ng/µl before labeling.
- Maintain the DNA in single-stranded form is required to ensure a good labeling efficiency.
- Enzyme labeled probes should not be denatured prior to addition to the hybridization buffer.

Preparation of Labeled Probe

KIT: AlkaPhos Direct labeling reagents from Amersham Pharmacia Biotech UK limited, Cat number: RPN 3680. CRR-star detection reagent Cat number: 3682 from Amersham.

1. Preparation of cross-linker working solution from kit: 10 µl of cross-linker working solution are need for each probe; thus for 3 probe, total is 30 µl. Take 8 µl cross-link tube (black tube in kit) + 32 µl H_2O supplied = 40 µl cross link working solution. (This cross-linker working solution can be saved at 2-8 C for later use.)
2. Dilute DNA to be labeled to a concentration of 10 ng/µl using the water supplied (10 µl = 100 ng DNA).
3. Place 10 µl of the dilute DNA sample in a microfuge tube and denature by heating for 5 min in a vigorously boiling water bath.
4. Immediately cool the DNA on ice for 5 min. Spin briefly in microfuge to collect the contents at the bottom of the tube.
5. Add 10 µl of reaction buffer (red tube in kit) to cooled DNA. Mix thoroughly but gently.
6. Add 2 µl labeling reagent (yellow tube). Mix thoroughly but gently.
7. Add 10 µl of the cross-linker working solution. Mix thoroughly. Spin briefly in microfuge to collect the contents at the bottom of the tube.
8. Incubate the reaction for 30 min at 37 C.
9. The probe can be used immediately or kept on ice for up 2 h. For long-term storage probes may be stored in 50% (v/v) glycerol at - 15 C for up to six months.

Hybridization

1. Pre-heat the required volume of prepared Alk. Phos direct hybridization buffer to 55 C. The volume of buffer should be equivalent to 0.25 ml/cm^2 of membrane (generally, about 23-25 ml per hybridization tube).
2. Place the blot (membrane) into the hybridization buffer and pre-hybridize for at least 15 min at 55 C in shaking water bath or hybridization oven.
3. Add the labeled probe to the buffer used for the pre-hybridization step (directly to the hybridization tube). Typically use 5-10 ng of probe/ml of buffer. (100-200 ng of probe for 20 ml of hybridization solution).
4. Hybridize at 55 C overnight in shaking bath or hybridization oven.

Post-Hybridization Stringency Wash

1. Primary wash in hybridization oven:

- Pre-warm the primary wash buffer at 55 C for general probe (low stringency) or 60- 65 C for species-specific probe (high stringency).
- Place blots in 1-2 ml/cm (30-40 ml) wash buffer.
- Wash for 2X 20 min.

2. Second wash
   - Prepare second wash buffer (100 ml in tray: 5 ml of 20X stock + 95 ml H_2O + 200 µl $MgCl_2$)
   - Wash bath at room temperature for 2X 10 min, gently agitating.

Solutions for Hybridization

A) Hybridization Buffer:

- Hyb Solution 500 ml bottle (in the kit).
- NaCl 14.61 g
- Blocking reagent 20 g (Kit)

Store in aliquots at - 20 C

(Add NaCl to Hyb buffer to give final solution of 0.5 M. Add blocking reagent to give a final concentration of 4 %. Mix at RT for 1-2 hours on magnetic stirrer until clear.)

B) Primary Wash:

- Urea 60 g
- SDS 0.5 g
- 0.5 M NaH_2PO_4 50 ml*
- NaCl 4.35 g
- 1.0 M $MgCl_2$ 0.5 ml
- Blocking reagent 1 g (kit),

Make up to a volume of 500 ml, can be stored 1 week at 4 C.

* monobasic sodium phosphate, adjust to pH 7.0 with NaOH.

C) Secondary Wash:

500 ml 20X Stock:

- Tris base 60.5g
- NaCl 56 g

Add above to 450 ml dH_2O, adjust pH to 10.00 with NaOH.
Store 4 months at 4 C

Secondary Wash, 500 ml Working Solution:

- Dilute 1:20, (20X stock, secondary wash stock) 25 ml
- dH_2O 475 ml
- 1M $MgCl_2$ 1 ml

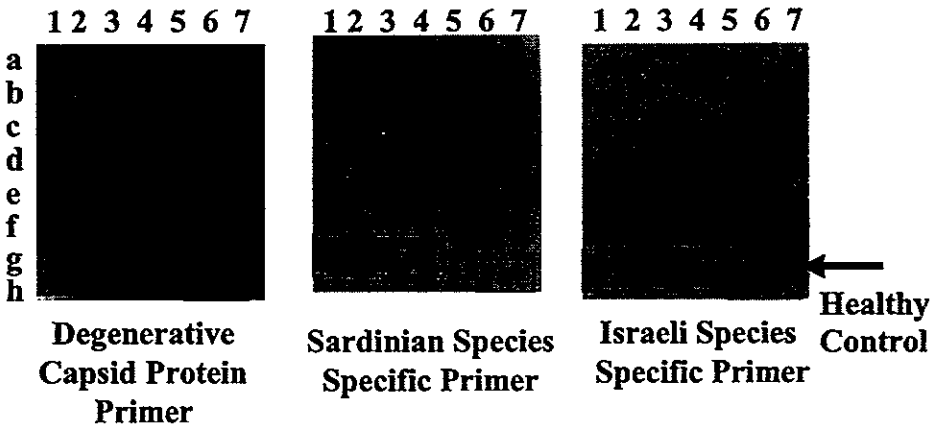
Signal Detection with CDP-Star

First, wear powder-free gloves or rinse gloves hands with water.

1. Drain excess secondary wash buffer from the blots by touching the corner of the blots against a clean surface, place the blots (sample side up) on clean non-absorbent, flat surface, eg, a tray. Do not allow the blots to dry out.
2. Pipette detection reagent (in the kit, CDP star) on to the blots, about 30-40 $\mu l/cm^2$ (2-4 ml/blots, enough to cover the whole blot) and leave for 2-5 minutes at room temperature. Drain excess reagent from membranes before next step.
3. Wrap the blots in Saran Wrap or place in detection bag. Place the blots DNA side up in the film cassette.
4. Switch off the lights and place a sheet of autoradiography film (Ex. ECL film, Hyper-film) on the top of the blots (shiny side of the film up). Close the cassette and expose for 4 h at room temperature. The DNA side of the filter (wrapped in saran Wrap) must be placed nest to the film for maximum sensitivity.
5. Remove the film and develop, you can also expose a second film for 24 hours.

Results

DNA Spot Hybridization



This example above is from tomato samples collected in Morocco and hybridized with the general probe (CP gene), TYLCSarV-species probe, and TYLCV (Israel)-species probe (PTYv21/PTYc287, primers). Three identical membranes were prepared. None of the probes hybridized with the DNA extracted from symptomless (non-virus infected) tomatoes, and general probe hybridized with most samples. Many tomato samples had mixed infections and more tomatoes were infected with TYLCSarV than TYLCV.

Discussion

This non-radioactive has been tested in laboratories in San Carlos University, Guatemala, Agricultural Research Center, Giza, Egypt by our group and has worked very well. This method offers several advantages. Many samples can be tested and the species-specific probes are effective in distinguishing between TYLCV and TYLCSarV, where as the general probe hybridizes effectively with these two viruses. Similar methods have been developed by Potter et al. (2003) for detection of bean-infecting begomoviruses.

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Confirmed by

Updated: November 5, 2002

TECHNICAL SHEET no. 11

VIRUSDETECTION: VARIABILITY ANALYSIS OF PVY STRAINS

GENERAL

Virus detected: PVY strains from tomato, potato and pepper samples

DEVELOPED BY

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Date: October 25, 2002

GOALS

Rapid detection of PVY strains from large number of samples, and analysis of the molecular variability of full genome.

INTRODUCTION

PVY is a member of the *Potyviridae*, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm). Currently, three main strains of PVY are know. (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is designed the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^O. (iii) PVY^N was recognized in the 1950's as causing serve veinal necrosis in tobacco. I was originally described from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^C strains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. There is, currently, consider effort to better understand the relationship between the different strains of PVY, there genomes and biological properties.

MATERIALS and METHODS

Tissue Blotting

PVY in field samples was primarily detected by tissue blotting (Lin et al., 1990). Leaf samples were directly imprinted on a nitrocellulose membrane (Protran, Schleicher et Schuell) by pressing a freshly cut section of the leaf mid-rib. The membrane was then air dried at room temperature and, if needed, stored at room temperature until processed. After saturation of the membrane for 30 min at room temperature in blocking buffer (0.35 M NaCl, 10 mM Tris-HCl pH 7.4, 1% (w/v) gelatin), the membrane was incubated for 2 h at room temperature with alkaline phosphatase-conjugated PVY-specific IgG's derived from a polyclonal antiserum, diluted in RIA buffer (10 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.1% (w/v) SDS and 1% (w/v) Triton X-100) (conjugate diluted 1/1000, similar to the concentration used for a DAS-ELISA assay). After three 10 min washes in RIA buffer, the membrane was incubated in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 9.5) supplemented with NBT and BCIP. Color development was stopped by washing the membrane in tap water and drying. Tissue blots were individually scored by eye or by observing the membrane under a stereo-microscope at low magnification.

Nucleic acid extraction

Method 1.

Sample leaves were ground (1/5, w/v) in PBS-Tween buffer (8 g of NaCl; 0.2 g of KH_2PO_4; 2.9 g of Na_2HPO_4 ($12H_2O$) and 0.5 ml of Tween 20 per litre) containing 2% (w/v) polyvinylpyrrolidone K25 and 20 mM DIECA. After centrifugation at 13,000 rpm for 10 min, 200 µl of supernatant were transferred to a microfuge tube, mixed with SDS to 1% (w/v) final concentration and incubated at 55°C for 15 min. One hundred microliters of 3 M potassium acetate were added, the mixture vigorously vortexed and incubated on ice for 5 min. After centrifugation (5 min, 13,000 rpm, 4°C), the supernatant was adjusted to 4.2 M NaI. Five microliters of a suspension of silica particles (Sigma) were added, carefully mixed by low speed vortexing and the mixture incubated at room temperature for 5 min. After a brief centrifugation (1 min, 5,000 rpm at room temperature) the supernatant was discarded and the pellet gently resuspended in 500 µl of washing buffer (20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 100 mM NaCl; 50% (v/v) ethanol). The centrifugation was repeated and the pellet of silica particles similarly washed twice more, before being resuspended in 400 µl sterile water. After incubation at 55°C for 5 min and centrifugation at 13,000 rpm for 2 min, 300 µl of the supernatant were transferred to a new tube. Total nucleic acid extracts were then used directly for RT-PCR amplification or stored at –20°C until used.

Method 2.

Total RNA extractions from PVY-infected plants were carried out with the basic phenol/detergent mixture (Robaglia et al., 1993). Leaf tissue (200 mg) was ground in a microcentrifuge tube with 0.3 ml of TE3D buffer/water saturated phenol mixture (1:2) containing 2% of beta-mercaptoethanol. [TE3D buffer: 2M Tris (pH not adjusted), 200 mM EDTA, 15% lithium dodecyl sulfate, 10% sodium deoxycholate, 10% Nodidet P40]. 0.4 ml of chloroform-isoamyl alcohol and 0.25 ml of 3 M ammonium acetate were added and the tube was vortexed for 1 min, then centrifuged for 15 min at 12-15 krpm. Then, 0.2 ml of supernatant was collected and mixed with 0.8 ml of 3.6 M LiCl, 10 mM EDTA, RNA was allowed to precipitate on ice for 1 h. The RNA was centrifuged (30 min at 12 krpm) washed with 70% ethanol and resuspended in 0.1 mM EDTA, pH 8, or DEPC-treated water.

RT-PCR

Method 1: amplification of Coat protein region

PVY-specific primers used were the sense primer corresponding to nucleotides 8504-8522 of the PVY genome (Robaglia et al., 1989) and the antisense primers complementary to nucleotides 9400-9418 of the PVY genome. RT-PCR was performed using the one buffer, one tube format. Briefly, 3 µl of total RNA extract were submitted to amplification in a 50 µl RT-PCR reaction mix [10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 50 mM KCl; 0.3 % (v/v) Triton X100; 250 µM of each dNTPs; 1 µM of each primer pair; 2.5 µl of formamide; 0.25 units of AMV reverse transcriptase (Stratagene) and 0.5 units of *Taq* DNA polymerase (Appligen-Oncor]. The mix was overlaid by 50 µl of mineral oil and the tubes were incubated for 45 min at 42°C for reverse transcription, followed by incubation for 5 min at 95°C for denaturation of RNA-DNA hybrids and reverse transcriptase. 35 cycles of amplification with the following thermal profile were then performed: 2 cycles (50 sec at 92°C, 50 sec at 37°C and 1min at 72°C) followed by 33 cycles (50 sec at 92°C, 50 sec at 45°C and 1 min at 72°C). Finally 10 min at 72°C were performed. PCR products were finally analyzed by electrophoresis in 0.8% agarose gels in TBE buffer and visualized under UV light following ethidium bromide staining.

Method 2: Full-length cDNA synthesis

The first-strand cDNAs were synthesized with AMV reverse transcriptase (promega) using viral RNA or total RNAs as templates. 1-5 µg viral RNA were mixed with 40-100 ng of antisense primer: 5'GCGTTAATTAATTTTTTTTTTTTTTTTTT-TTTTTTTTTTTGTCTCCTGATTGAAGTTTACAGTC3'in water and heated 2 min at 70°C. Nucleotides (1 mM Final concentration of each), 20 units of RNAse inhibitor and 40 units of ANV-RT (Promega) were added to the 20 µl reaction containing 5X RT-Buffer. After 1 h at 42°C , the samples were diluted 20-100 times with water. The cDNAs were amplified using the following polymerase mixes: rTth and Vent DNA Polymerase (XL-PCR Kit, Perkin Elmer). Reaction conditions were as recommended by the suppliers: 0.2 mM dNTPs, 200 ng primers, 1.1 mM $Mg(OAC)_2$, 1X buffer, 4 units of enzyme mix. 1 µl of diluted cDNA template was used with antisense and sense primer: 5' CTGCCGCGGATTTAGGTGACACTATAGAAATTAAAACAACTCAA-TACAACATAA 3'. PCR was run using the following program: initial denaturation at 94°C for 1 min, 16 cycles of 15 sec at 94°C, 10 min at 65°C and 12 cycles of 15 sec at

94°C and 10 min at 65°C with and added 15 sec at each cycle, followed by final 10 min elongation at 72°C.

An aliquot of the PCR product was analyzed by 0.8% agarose gel electrophoresis.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF cDNA

The amplified cDNA was purified from the reaction mixture either by phenol-chloroform extraction and ethanol precipitation or by gel electrophoresis excision of the appropriate band and isolation of the cDNA using the Gene Clean Kit. One µg of cDNA was digested with restriction enzyme of choice: *Bst*YI, *Hae*III, *Hinc*II, *Pvu*II. The digestion products were analyzed on 1% agarose gel.

RESULTS & DISCUSSION

The tissue-blotting assay proved simple, fast, reliable and very economical. As previously reported this simple technique offers numerous advantages when a large number of samples need to be processed rapidly. In this respect, this technique should prove, in the future, very useful for epidemiological studies of PVY. The only key parameter seems to be the quality of the antiserum, which causes interpretation problems, if it gives a significant cross-reaction to healthy plant components.

Coat Protein gene amplification by RT-PCR (fragment of 870 bp) is reliable and very rapid protocol for detection of most of the PVY strains spreading in different hosts: pepper, tomato and potato.

RFLP scanning of the whole genome could be seen as a fast highly informative and relatively simple supplement for the established methodologies. Such a fast method will be of interest for analyzing viral population structures and their evolution under different pressures created by local production practices.

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Confirmed by

Edited by D. P. Maxwell, Oct. 28, 2002

TECHNICAL SHEET No. 12

VIRUS DETECTION: PVY, PVX and PLRV

MULTI PLEX – RT-PCR

General

Virus detected: PVY, PVX and PLRV in one reaction
General method: multiplex-RT-PCR

Developed by

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Address (E-.mail): aashalaby@intouch.com

Date: April 11, 2002

Goals

The m-RT-PCR assays developed is a reliable, rapid, and sensitive method for the detection of these three viruses in one reaction. The use of the m-RT-PCR assays are recommended for applications where they improved the sensitivity over standard RT-PCR and are necessary for the early detection of infection and for quarantine and breeding programs.

Introduction

Potato *(Solanum tuberosum* L.) often becomes infected with two to three different viruses. The most common viruses affecting potato crops throughout the world are *Potato virus Y* (PVY, potyviruses), *Potato virus X* (PVX, potexvirus) and *Potato leaf roll virus* (PLRV, luteovirus). Egypt imports all potato seeds for planting from Europe, where theses viruses are present.

The current method routinely used for detection of these viruses is ELISA and there are no procedures to detect these three viruses in one step reaction. Multiplex-RT-PCR for PVY strains has been reported by Nei and Singh (2002).

Materials and Methods

Viral RNAs were extracted from potato samples infected with potato viruses (PLRV, PVX and PVY) using Rneasy Plant Mini kit (QIAGEN® cat # 74903) according to the follows:

1. Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer to an appropriately sized tube.
2. Add 450 µl of either Buffer RLC to a maximum of 100 mg of tissue powder. Vortex vigorously. A short (1-3 min) incubation at 56 C may help to disrupt tissue.
3. Apply lysate to the QIAshredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer the flow-through fraction from QIAshredder to a new tube without disturbing the cell-debris pellet in the collection tube.
4. Add 0.5 volumes (usually 225 µl) ethanol (96-100%) to the cleared lysate and mix well by pipeting.
5. Apply sample (usually 675 µl), including any precipitate, which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at 10,000 rpm.
6. Pipet 700 µl Buffer RWI onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow-through and collection tube.
7. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500 µl Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm. Discard flow-through and reuse the collection tube in step 8.
8. Add 500 µl Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane.
9. Transfer RNeasy column into a new 1.5-ml collection tube, and pipet 30~50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 10,000 rpm to elute the RNA. Repeat if the expected RNA yield is >20 µg.

Design of Primers

In order to detect PVY, PVX and PLRV in one-step reaction, we all with M. K. Nakhla (University of Wisconsin-Madison) designed three specific primers for each virus to amplify the full-length of the coat protein gene. The viral sequences were retrieved from GenBank and compared.

Table (1) The oligonucleotide sense and complementary sense primer pairs used for m-RT-PCR and RT-PCR

a- The oligonucleotide sense and complementary sense primer pairs used for m-RT-PCR			
Primer name	Nucleotide sequence (5' to 3')	Polarity	Expected amplicon size (bp)
Potato virus Y PVYCPv*BamHI* PVYCPc*EcoRI*	5' TCAAGGATCCGCAAATGACACAATTGATGCAGG 3' 5' AGAGAGAATTCATCACATGTTCTTGACTCC 3']	sense comp.	801 ~ bp
Potato virus X PVXCPv*EcoRI* PVXCPc*NcoI*	5'-GATAGAATTCAGATGACTACACCAGCCAACACC-'3 5'-TACGCGTCGGTTCCATGGACGTAGTTATGG TGG-'3	sense comp.	700 ~ bp
Potato leaf roll virus PLRVCPv*EcoRI* PLRVCPc*NcoI*	5'AATAGAATTCTAATGAGTACGGTCGTGGTTARAGG 3 5'AAAACCATGGCTATYTGGGGTTYTGCARAGCYAC-3	sense comp.	548 ~ bp

Multiplex reverse transcription polymerase chain reaction (m RT-PCR)

Three primer mixes (Table 1) were made up with each primer at a final concentration of 0.45 µM. The primer mixes could be stored frozen at -20°C, or freeze-dried for resuspension to the same concentration at a later date.

The one step RT-PCR kit and protocol (QIAGEN) was used according to Manufacturer's instructions as follows:

Fifty nanograms of viral RNA was mixed with 10 µl of 5x buffer, 2 µl of dNTPs mix, 10 µl of 5x Q-solution, 3 µl of 10 µM of each viral sense primer, 3 µl of 10 µM of each complementary sense primer, 2 µl of QIAGEN enzyme mix, 0.2 µl of RNase inhibitor and water up to 50 µl.

The one step RT-PCR mixture varied depending on the number of intended viruses to be detected and the number of primers involved.

The PCR parameter starting with 30 min at 50°C and 15 min at 95°C (for RT Reaction) followed by 30 cycles, each cycle consisted of denaturation at 94°C (1 min), primer annealing at 55°C (1 min) and extension at 72°C (1 min), with final step for 10 min at 72°C.

The reaction products were subjected to agarose gel electrophoresis.

Results

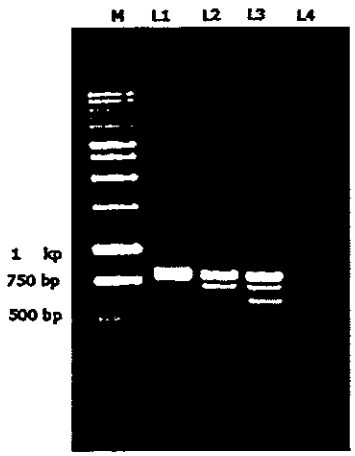

Fig. (1.): Detection of PVY, PVX and PLRV by RT-PCR and multiplex-RT-PCR from cDNAs primed by specific sense and complementary primers for each virus as shown in Table 1. Lane M, DNA leader molecular size markers; the sizes in bp are indicated on the left hand margin. Lane 1, amplified PVY by RT-PCR, lane 2, multiplex RT-PCR in which two pairs of viral specific primers for PVY and PVX were present in the reaction and two viral RNAs, and lane 3, multiplex RT-PCR in which three pairs of viral specific primers for PVY, PVX and PLRV were present in the reaction and three viral RNAs. The amplified products were: PVY (800 bp), PVX (700 bp) and PLRV (550 bp).

DISUSSION

Multiplex PCR is increasingly used because it improves the efficiency of diagnostic PCR (Johnson, 2000). In the near future multiplex PCR will probably be adapted for the simultaneous detection of viruses of one particular crop and for the

simultaneous detection of other major plant pathogens such as viruses, viroids, bacteria, and fungi in the same reaction, as already demonstrated for viruses and viroids (Nie and Singh, 2001). An increase in sensitivity would probably be achieved, if multiplex PCR in a single closed tube were developed based on this technology.

These techniques demonstrate the feasibility of multiplex RT-PCR based on the specific primer design for the identification of several potato viruses i.e., PVY, PVX and PLRV, in a single step reaction. Such a method should increase both the sensitivity and specificity of the diagnosis and thus reduce the possibility of false negatives.

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CONFIRMED BY

TECHNICAL SHEET No. 22

VIRUS DETECTION: *Grapevine virus A* (GVA)

METHODS: RT-PCR, and IC-RT-PCR

General

Virus detected: GVA from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription PCR.

Developed by

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Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu

Date: Nov. 1, 2002

Goals

Sensitive methods for GVA detection in grapevines.

Introduction

Grapevine, which belongs to Vitidaceae family, is considered the second important fruit crop, after olives in Palestine. Based on the Palestinian Central Bureau of Statistics (1), the total fruit-trees cultivated area in Palestine is 1,118,075 dunums, of which 83,174dunums are grapevine yards (7.4%). About three quarters of the grapevine area is located in Bethlehem and Hebron districts (1).

Until recently, 44 different viruses have been identified in grapevines worldwide, not all of them cause serious diseases though (2). Viruses causing the most important grapevine diseases in Palestine belong to three genera; Nepovirus, Closterovirus, and Trichovirus. Another disease detected in Palestine (grapevine fleck) is caused by a virus that has not been grouped yet (2).

A preliminary work conducted in1995 (3) in an attempt to determine incidence of viral infections in viticulture in Palestine has shown that infection ranges between 50-98%. The most prevalent virus was GVA followed by Grape leaf roll association virus (GLRaV) 1,3, grapevine fleck virus, GLRaV-2, GVB, Grape fanleaf virus, and GLRaV-7. The highest infection with most viruses was in Bethlehem, Jenin, and Hebron areas. The GFLV and GLRaV-7 were the least common viruses among those detected in Palestine, and were restricted to Bethlehem, Jenin, and Hebron areas.

Grapevine A is a single stranded RNA virus belonging to the Trichovirus genus. GVA is one of the most common causes of the Rugose wood disease, particularly the syndrom known as Kober stem grooving disease (3). This disease is

highly distributed in Bethlehem and Hebron areas, where infections reach 85% (3). The virus was first reported in *Vitis vinifera*; from Taranto, Italy; by Ciccarone. The symptoms caused by GVA include pits and grooves in the trunk. GVA is transmitted by a vector (very rarely); an insect; Pseudococcidae. Virus is also transmitted by mechanical inoculation (only to *Nicotiana glutinosa*); or by grafting but not transmitted by contact between plants; neither by seed or pollen. Grapevine A susceptible host species are *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Vitis labrusca*, *Vitis rupestris, Vitis rupestris var. Rupestris, and Vitis vinifera* (4).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set was used to detect GVA in the tested samples:

Primers

PCR Product: 430 bp of the coat protein

Primer 1, which is a complementary sense primer:
C995: 5 AAGCCTGACCTAGTCATCTTGG 3

Primer2, which is the antisense primer: H587: 5 GACAAATGGCACACTACG 3

RNA extraction from grapevine stems and petioles for RT-PCR.

1. Cut the sample (0.5-0.7g of the stems or petioles) into pieces by a scalpel and grind in liquid nitrogen in a mortar.
2. Add 5 volumes the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudum till you get green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4°C.
5. Distribute the supernatant into aliquots and store at –80°C till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65°C for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, qsp with dH_2O).
9. Incubate at 100°C for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H_2O).
13. Incubate at 37°C for 1 h.
14. Store at –20°C till used in the PCR.

IC-RT-PCR:

Coating:

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 µl in each well of the ELISA plate.
2. Incubate the plate at 37°C for 2 h.

Extraction: (all the steps should be done at 4°C)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and grind in the presence of carborudum.
3. Collect the homogenate and centrifuge at 10,000 rpm for 10 min at 4°C.
4. Transfer the supernatant into a new tube.
5. At the end of the coating period, deposit 100 µl of the extract (supernatant) per each well.
6. Incubate the plate at 4°C for overnight.

RT reaction:

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, 1% Triton X-100, enough dH_2O as required) and heat at 65°C for few min.
7. Wash the plate 3 times with PBST.
8. Add 20 ml of the heated RT mix per each well, then incubate the plate at 65°C for 15 min.
9. Put the plate on ice and release the virus by 5-10 sec pipetting liquid in each well, then transfer the well content to a microfuge tube.
10. Incubate the tubes at 100°C for 2 min, then at 4°C (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5 µl of dH2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37°C for 1 h.
13 Store at –20°C till use for the PCR.

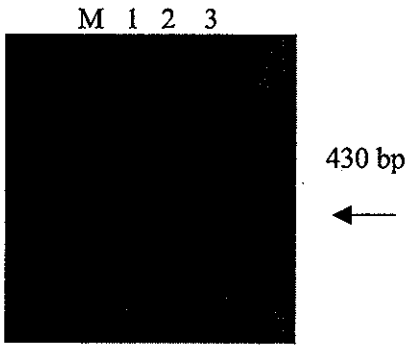
PCR conditions:

- 5 µl of the cDNA, 0.2 mM dNTPs, 1X Polymerase buffer including 2.5 mM $MgCl_2$, 100 ng of each primer, and 1 unit of the *Taq* DNA polymerase. Complete to 50 µl with dH_2O. However, amounts less than 1.25 units of the *Taq* DNA polymerase failed to detect GVA. Hence we used 1.25 units per reaction for detection GVA.

PCR cycles:

(Hot start: Add the *Taq* DNA polymerase while the reaction is heated at 94°C): 94°C for 5 min, then 5 cycles of 94°C for 50 sec, 50°C for 1 min, and 72°C for 1 min. 30 cycles of 94°C for 50 sec, 51°C for 1 min, and 72°C for 1 min. A final step of 72°C for 5 min.

Results:



M: molecular weight marker.
1: IC-RT-PCR product of tested sample.
2,3: RT-PCR products of tested samples.

Comments

The detection of GVA using the IC-RT-PCR method was much sensitive than the RT-PCR method. The results showed very faint bands in some of the tested sample using the RT-PCR method compared to the bands detected using the IC-RT-PCR method. Hence, we recommend the later technique for the detection of GVA especially in samples containing low titer of the virus, which could be previously determined by ELISA or other tests.

References:

1. Palestinian Central Bureau of Statistics. Agricultural Statistics 1995/1996.

2. Hadidi, A., Khetarpal, R. and Koganezawa, H. (1998). Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, MN

3. Ra'ed Al-Kowni. 1997. Institute agronomique Mediterraneen De Bari. M. Sc Thesis.

4. http://life.anu.edu.au/viruses/Ictv/

Confirmed by

Technical Sheet no. 25

IC-RT-PCR for the detection of *Grapevine Leafroll virus 1,3*

Goal

Detection of GLRV 1,3 by one step IC-RT-PCR.

Developed by

Name of researchers: Ghandi Anfoka, Al-Balqa' Applied University, Al-Salt, Jordan
Address (Email): anfoka@joinnet.com

Date: Nov. 27, 2002

Introduction

Leaf roll is a vitis disease with a very important economic impact throughout the world. It is characterized by a down rolling of leaves and reddening or yellowing of limbs and its occurrence causes significant reduction in crop quality and quantity. Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) is a powerful tool for diagnosis of grapevine viruses.

Materials and Methods

Tissue extraction

Leaf tissues from grape plants showing disease symptoms were collected from the University research center and kept at 4°C. Using pestle and mortar, 0.5 g of leaf petioles were cut into small pieces and grinded in 2.5 ml extraction buffer. The sap was transferred into an eppendorf tube and centrifuged at 14,000 rpm for 1 minute and supernatants were kept on ice.

IC-RT-PCR

Coating with antibodies:

- Polyclonal antibodies, developed by Sediag (France) were diluted using coating buffer (carbonate bicarbonate). Three antibody dilutions were tested (1:1000, 1:500 and 1:250).
- Two hundred microliters of diluted antibody were deposited into PCR tubes and tubes were incubated at 37°C for 4 h.
- Tubes were washed three times with PBS-T and briefly centrifuged to remove the remaining droplets of buffer.
- Two hundred microliters of leaf sap were dispensed into PCR tubes and incubated overnight at 4 °C.
- Tubes were washed three times with PBS-T.

- Tubes were incubated at 65°C for 10 min and used for RT-PCR.

One step RT-PCR

Primers:

The primer set of GLRaV 3 designed by Saldarelli and Hadidi (1994) was used:

Downstream primer (C547): 5'- ATT AAC TTG ACG GAT GGC ACG C- 3'.

Upstream primer (H229): 5'-ATA AGC ATT CGG GAT GGA CC -3'.

Length of the predicted product is 340 bp.

RT-PCR kit:

The access RT-PCR system kit developed by Promega (cat # A1260) was used in this study according to the manufacturer's instructions.

RT-PCR mixture:

- RT mixture per tube consisted of: 26 µl nuclease-free water, 10 µl AMV/Tfl buffer (5X), 5 µl antisense primer (10 µM), 5 µl sense primer (10 µM), 1 µl dNTP (10 mM), 2 µl $MgSO_4$ (25 mM), 0.25 µl AMV reverse transcriptase (20 U/µl) 1 µl Tfl DNA polymerase (5 U/µl). Tubes were vortex and then centrifuged for few seconds.

PCR cycles:

PCR cycle previously described by Acheche et al. (1999) was used. It consisted of: 46°C for 60 min, 94°C for 5 min then 30 cycles of 94°C for 30 sec, 56°C for 45 sec, and 72°C for 1 min, and a final step of 72°C for 10 min.

Agarose gel electrophoresis

PCR products (8 µl) were electrophoresed on 1% agarose gel and stained with ethidium bromide before gel was photographed under UV illumination. Lambda DNA *Eco*RI/*Hin*dIII digest was used to determine the size of amplified product.

Results

Part of the RNA polymerase gene (340 bp) of GLRV 1,3 could be amplified using one step IC-RT-PCR. The expected size of PCR (340 bp) could be only detected in tubes coated with antibodies diluted 1:250 and 1:500 (Fig. 1).

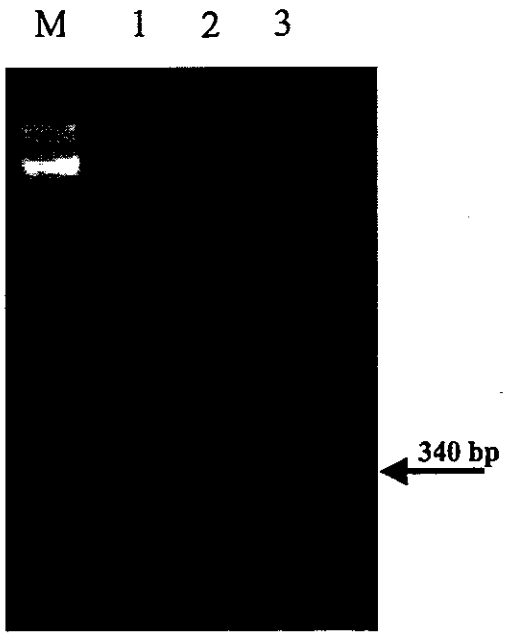


Figure 1. IC-RT-PCR of a grape sample. M: lambda DNA/*Eco*R1+*Hin*dIII marker. 1: tubes coated with antibodies diluted 1:1,000, 2: diluted 1:500, and 3: diluted 1:250.

Comments

This technique is sensitive, saves time and efforts and can be easly applied to detect GLRV 1,3 in large number of samples without the need of RNA extraction.

References

Acheche, H., Fattouch, S., M'Hirsi, S., Marzouki, N., and Marrakchi, M. (1999). Use of optimised PCR methods for the detection of GLRaV3: a closterovirus associated with grapevine leafroll in Tunisian grapevine plants. Plant Molecular Biology Reporter 17:31-42.

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Confirmed by:

Date: Dec. 4, 2002

TECHNICAL SHEET no. 31

VIRUS DETECTION: *Cucurbit yellow stunting disorder virus* (CYSDV) by RT-PCR

General

Virus detected: cucurbit yellow stunting disorder virus (CYSDV) from cucurbit leaves

Method: RT-PCR

Developed by

Name of researcher: Dr. Yusuf Abou-Jawdah, American University of Beirut, Lebanon
Address (email): abujawyf@aub.edu.lb

Date: October 26, 2002

Goals

Developing a reliable and fast detection system for CYSDV.

Introduction

Cucurbit yellow stunting disorder virus (CYSDV) is a member of the genus *Crinivirus* within the family *closteroviridae* (Fauquet and Mayo, 1999). CYSDV has a bipartite single-stranded plus-sense RNA encapsidated in long and flexuous particles. It is a whitefly-transmitted viral disease of cucurbits. It was first reported by Hassan and Duffus (1991) in the United Arab Emirates. This virus was also reported in Europe (Celix et al. 1996). Rubio et al. (2001) studied 71 isolates from collected from Jordan, Lebanon, Saudi Arabia, Spain, Turkey and North America. They found that these isolates could be divided into two subpopulations. Symptoms start as interveinal mottle on the middle leaves and develop into severe yellowing as leaves grow older. The fruits look normal but the fruit set is greatly reduced leading to a yield loss of about 50% (Abou-Jawdah et al., 2000). The development of an RT-PCR system would provide an easy, reliable and quick method to detect the disease.

Materials and Methods

1. Sample collection:

Samples of infected cucurbit leaves (cucumber, squash, melon) were collected from greenhouses in the coastal area of Lebanon, at the end of the growing season, when symptoms are most apparent. Generally, the 4th or 5th yellow, papery leaf from the top of the plant was collected as recommended in the literature.

PREVIOUS PAGE BLANK

2. Total RNA extraction:

- 0.2 g of leaf tissues are ground in liquid nitrogen
- Add 400 µl of 100mM Tris-HCl (pH 8) containing 0.2% sodium dodecyl sulfate (SDS) and 10 mM EDTA
- Extract RNA with 1 volume of (1:1) phenol:chloroform
- The aqueous phase is then adjusted to 2 M LiCl and incubated overnight at 4°C
- Centrifuge at 12,000g for 15 min and dissolve the pellet in 100 µl RNAse-free-water

3. Primer design:

Using the NCBI gene bank, the nucleotide sequence of the CYSDV HSP70 gene (AJ223619) was retrieved and used for primer design (Celix et al., 1996). In the sequence below, the bolded areas are the parts used for primer design.

```
   1 atggcgaagg ctggtttgga gtttggtact actttctcta ctatcagcag ttatgttaat
  61 ggtgttatga aagttttgaa attgaacgaa actgaattta tccctacctg tttagccata
 121 acgtctaata atgatgtggt tgttggaggt cccgctcaag tattgtctaa tagtgacatg
 181 cctaactgtt acttttatga cttgaaaagg tgggtaggtg ttgacagtat caattataat
 241 gtgataaaaa ccaaaatcaa tccagcgtat gtcaccgagt tacgtggtaa cgacgtgtac
 301 atcactggta tcgatagagg ttatacctgc acttacacag tcaaacaatt gatattgtta
 361 tacattgaaa ctttagtaag attgttttct aaagttgagt ccataaccat aactagtctt
 421 aacgtctctg ttccagctga ttataaatgt aagcagcgta tgtttatgaa atcagtttgt
 481 gacagtctag gtttttcatt acgtagaatt ataaacgaac catctgcagc agctatatac
 541 tttgtttcaa agtatccgca gtataacaac ttcctgatgt atgacttcgg aggaggaact
 601 tttgattctt ctctaatagt cagagacggt aagtatgtca cagtggctga tactgaaggt
 661 gattcgtttt tgggaggtag agacattgat aatgctatcg ctgactatat aacaacgacg
 721 tatggtatga aaggtggttt gtccgctgac gtactggcgt ctataaagga ggattgtaat
 781 tctaagggta gagagaattt caatgttata gattcatcag gcaaacttca taatgtgaaa
 841 ttcacaagac aagatctgag tcgttgcatt gaacctttct ctaagaagag catagcactg
 901 cttgataata tggtagtgcg taacataaca aaagattccg ctgtgtttat ggttggaggt
 961 tcatcattgt tgaagaaagt tcaacatgat gtgatgaatt actgtgctag gacgaaacta
1021 gaatgcatca ttgataaaga ccttagatca gcagtgtcat ttggttgctc tatgtcacat
1081 gcccaagaag acacaaaaaa tatgatatac atcgattgta attcacatcc cttaatgggc
1141 atatcatatt tttgttctcc aaagattata gttaggaaac ctgtggccat accttatact
1201 ggagtgcgag aagaaacctt aacaagacac tatacaattt tgaacgttta cgagggatca
1261 gatcccttcg ttttgaacaa cgattggttg attagtgcca atatgcagtc caacaagtac
1321 ggtgagatag gtgatacatt acaatatctt tacaaatata atgtagatgg catcttagag
1381 ttggttgtga ggaataaaag aacaggtaag gagacggtac ttccaattcc tttgcttttg
1441 actgagagta taaagaagct ggatgtaaat ctaactcaat tgtcaaatat tgatgaattg
1501 gcaactttgg tagctattat gagttactat aagcctgaat taaagtacct cctgacttat
1561 gtaaagacac caactatttt tgaaaatgaa attaagaaat ttggatctgg tgaagattta
1621 tataaatcct tagctgctct gaataaaaat ttcaagtaa
```

Primer 1: 5' TTG GGC ATG TGA CAT 3' (410L)

Primer 2: 5' AGA GAC GGT AAG TAT 3' (410U)

4. RT-PCR using the "Access RT-PCR system" (Promega):

PCR mix: 20 µl reaction mixture containing 80 ng of primers and 4 µl of RNA sample to each bead.

PCR cycles:

- 1 cycle at 48°C for 45 min
- 1 cycle at 94°C for 2 min
- 35-40 cycles at 94°C for 30 sec, 42°C for 30 sec, 68°C for 70 sec
- 1 cycle at 68°C for 7 min

- 4°C end of program

The PCR product was visualized by electrophoresis in 1.2% agarose gel in TAE.

Results

Electrophoresis from extracts of virus-infected cucumber, melon and squash plants showed a specific DNA of about 460 bp that was absent in extracts of the respective healthy controls.

Discussion

This RT-PCR technique provides an easy and consistent procedure for CYSDV detection in plants with yellowing symptoms.

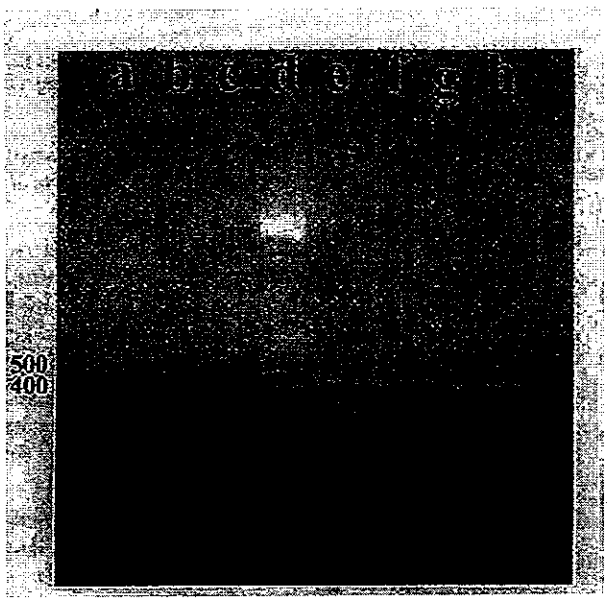

Figure 1. Gel electrophoresis of RT-PCR amplified products in 1.2% agarose gel.
Lanes a-c: healthy cucumber, melon and squash, respectively; lane d: 100 bp ladder (Pharmacia); Lanes e & f: infected cucumber, lanes g & h: infected melon and squash, respectively.

Literature Review

Abou-Jawdah, Y., H. Sobh, A,. Fayyad, H., Lecoq, B., DelEcolle, and Trad-Ferre, J. 2000. Cucurbit yellow stunting disorder virus – a new threat to cucurbits in Lebanon. Journal of Plant Pathology 82: 55-60.

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