PN-ABL-462

9.198

FINAL REPORT

Covering Period: June 30, 1989 - June 30, 1994

Submitted to the Office of the Science Advisor
U. S. Agency for International Development

Cloned DNA to Study Molecular Biology, Host-vector Relations and Control of Maize Rayado Fino Virus

Principal Investigator: Dr. Pilar Ramirez de Baudrit
Grantee Institution: University of Costa Rica

Collaborator: Dr. Rosemarie W. Hammond
Institution: U.S. Department of Agriculture-Agricultural Research Service

Project Number: 9.198
Grant number: 936-5542.13

A. I. D. Grant Project Officer: David Losk/Flora Ruiz

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Executive Summary

Maize rayado fino virus (MRFV) is of great agronomic importance as it produces significant yield losses in maize throughout the Central and South Americas. The aim of this project was to gain a better understanding of the virus and how it causes disease in maize in order to design strategies for control of the disease. The virus is insect transmitted, yet control of the disease by insecticides is ineffective, costly, and ecologically dangerous.

Through our studies, we have identified and obtained the viral gene encoding the coat protein. This gene will be extremely useful in the creation of virus resistant plants by genetic engineering, and a collaboration with Mexican scientists has been made to engineer maize with this gene. In addition, we have identifed resistant/tolerant maize accessions from the maize collection at CIMMYT. These accessions will be useful in the future for introducing virus resistance into locally grown cultivars by classical breeding. We have also performed the most comprehensive survey of the incidence and distribution of MRFV in maize fields in Central and South America using molecular techniques. This data is useful to regulatory officials in making informed decisions regarding disease status, and to ecologists, breeders, plant pathologists and others whose work involves maize germplasm.

This research program has allowed the introduction of new technology into the LDC program by training of the LDC scientists in the U.S. laboratory. The planning and performing of experiments by the Costa Rican and U.S. laboratories has been truly collaborative and has yielded many interesting and useful results. Many molecular techniques that are essential to a plant pathologist, such as polymerase chain reaction (PCR), nucleic acid hybridization, cDNA cloning, and nucleic acid sequencing, have been acquired by the LDC through this collaboration and will greatly enhance their ability not only to diagnose plant diseases rapidly and reliably, but also to creatively develop means for control of the diseases. The infrastructure of the LDC institution was also improved by the building of a modern greenhouse, and the purchase of a freezer, cold chamber, water bath, and orbital shaker. In addition, several graduate students received their M.Sc. degrees while being trained and supported from this grant.

Section I. Research Objectives

The overall aim of the proposed research was to investigate the complex relationships between maize rayado fino virus (MRFV), its insect vector, and tolerant and susceptible cultivars of maize. In Costa Rica and many developing countries of Latin America, maize is one of the principal food sources. Although maize is a main source of food in these countries, yields are low, averaging 1.3 metric tons/ha, considerably less than the 3.1 tons/ha world average (CIMMYT, 1981). There is no doubt that MRFV contributes to the reduced yield. The incidence of MRFV infection generally varies from 0-40%, but in some areas it reaches nearly 100%. Our research on MRFV and its expression in its insect and plant host addressed important questions about the infection process which will aid in our goal of introducing resistance to MRFV into maize cultivars grown in this region of the world. Once obtained, resistant cultivars would be made available to LDC breeders.

The research approach had three objectives; the results are described in the next section:

1. The production of a more complete cDNA library of the MRFV genome and nucleic acid seqeuncing of these clones in order to determine the genome expression strategy of marafiviruses.

2. The use of available nucleic acid probes and antisera and the development of new nucleic acid probes and technology to study the replication, accumulation, and movement of MRFV in populations of tolerant and susceptible cultivars of maize.

3. The use of these molecular probes to study viral replication and accumulation in the insect host.

The project complemented a high priority research program at the University of Costa Rica aimed at the study of the cellular and molecular biology of viruses and other pathogens endemic to the tropics and important to man, cultivated plants, and domestic animals in Costa Rica. MRFV was discovered in Costa Rica by a Univerity of Costa Rica scientist (Gamez, 1969). Since its discovery, extensive studies of the epidemiology and ecology of the virus have been undertaken (Gamez, 1983). The physical characteristics of the virus were studied and described by Leon and Gamez (1981) and Gamez and Leon (1988), both at the University of Costa Rica. The molecular characteristics of the virus, such as its nucleotide sequence and genome expression strategy were, however, unknown at the time our study began. Historically, therefore, it was natural that these studies should take place at the University of Costa Rica, with technical support from an outside laboratory.

Organizational support came from the U. S. Department of Agriculture, Agricultural Research Service in providing the salary support for the co-PI and the laboratory space and equipment where much of the research was conducted. The University of Costa Rica

provided organizational and administrative support for the operation of the project as well as laboratory equipment, laboratory space, land on which the greenhouse was built, the salary of the PI and communication costs. BOSTID provided assistance to the project in the arrangement and funding for electron microscopy training for Federico Albertazzi in October 1991. This assistance was gratefully appreciated.

Section II. Methods and Results

Accomplishments that have not been published will described in some detail. Those that are either published, submitted, or in preparation are attached in Appendix II.

Objective 1. The production of a more complete cDNA library of the MRFV genome and nucleic acid sequencing of these clones in order to determine the genome expression strategy of marafiviruses.

Several cDNA libraries have been synthesized using MRFV genomic RNA as the template. In some cases, random primers have been used, in other cases, either polyadenylation followed by priming with oligo dT or alternatively, specific primers have been used to produce the first strand of cDNA. The result is that we have a number of cDNAs representing the complete MRFV genome ($3x10^6$ Mr; 6KB) (Figure 1). Some of these cDNAs overlap and we are in the process of aligning the cDNAs and completing sequence analysis. Preliminary sequence analysis and expression of portions of the genome as fusion proteins in Escherichia coli allowed us to identify the capsid protein gene at the 3' terminus of the RNA. Once the complete genome is assembled, we will be able to assign some functions to certain regions by comparisons with other plant RNA virus sequences, eg. the replicase gene.

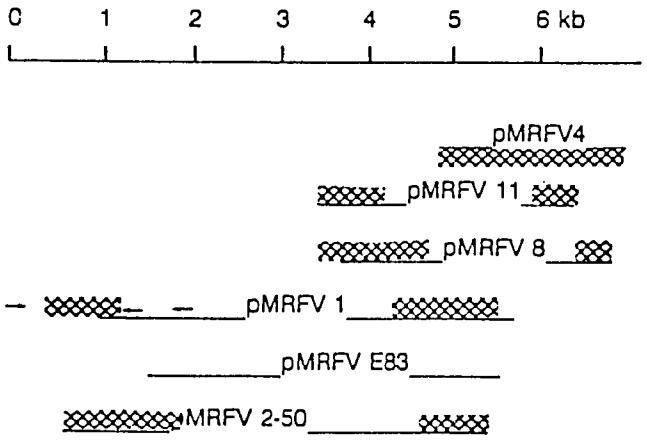


Figure 1. Schematic of cDNA clones of the MRFV genome. The major cDNA clones to be used for sequencing are shown relative to their location on the MRFV genome. The arrows indicate the positions of oligonucleotide primers to be used for obtaining the cDNA and ultimately, the sequence, corresponding to the 5' terminus of the RNA.
Shaded areas show the amount of cDNA sequence that has been obtained to date.

One of our most important discoveries has been the identification of the gene encoding the capsid protein of MRFV. Nucleic acid sequence analysis and in vitro expression of cDNA clones derived from MRFV as well as amino acid sequence analysis of the capsid protein has allowed us to identify the gene encoding the capsid protein of MRFV. The isolation and identification of this gene will allow us to consider the possibility of introducing resistance to MRFV in maize by the mechanism of coat protein expression. We have established a collaboration with the Department of Genetics, CINEVESTAV IPN - Unidad Irapuato, Mexico to introduce the coat protein gene contructs that we have made into maize and to provide training in maize transformation to a scientist at the University of Costa Rica.

Objective 2. The use of available nucleic acid probes and antisera and the development of new nucleic acid probes and technology to study the replication, accumulation, and movement of MRFV in populations of tolerant and susceptible cultivars of maize.

An additional use envisaged for the cDNA clones generated from the MRFV template was the sensitive, quantitative detection of the virus in its plant and insect hosts. To this end, we have developed radioactive, and more importantly non-radioactive, strand-specific probes from selected cDNA clones (Ramirez et al., 1990; Appendix II). Using these cDNA probes as well as highly specific monoclonal and polyclonal antibodies to MRFV (developed at the University of Costa Rica-CIBCM by Dr. Carmen Rivera), we have been able to follow the progression of MRFV infection in the plant host (Albertazzi et al., in preparation, Appendix II) and to assess the tolerance and susceptibility of selected maize cultivars and accessions obtained from CIMMYT (Mexico) to MRFV (Bustamante, et al., in preparation, Appendix II).

The pattern of accumulation of MRFV in susceptible Zea mays. L. var Guarare was analyzed after transfer of viruliferous leafhoppers (Dalbulus maidis) to healthy plants. Groups of 10 plants were exposed to the insects for an inoculation period of 3 to 4 days. After the insects were killed by spraying with insecticide, the individual leaves on the plants were monitored for symptoms and virus accumulation over a period of 7 to 12 days. As seen in Figure 2 (a-c)(page 7), the combined data obtained from observed symptoms, ELISA, and slot blot hybridization, respectively, viral RNA and capsid protein could be detected in asymptomatic leaves as well as symptomatic leaves. As can be clearly seen in tissue prints of infected plants (Figure 3,page 7), the pattern of accumulation of MRFV corresponds to the vascular tissue [see Albertazzi, et al., Appendix II].

Strand specific RNA probes as well as antisera were used to analyze the accumulation of virus in selected maize accessions obtained from CIMMYT. We were interested in determining if any maize accessions express tolerance or susceptibility to MRFV infection, and if so, at what level. Five hundred plants composed of twenty genotypes of maize (14 of which were obtained from CIMMYT and 6 Costa Rican genotypes) were analyzed for response to MRFV infection by assaying symptomatology, the level of capsid protein and the level of viral RNA. Two groups of plants were identified based upon these assays - one

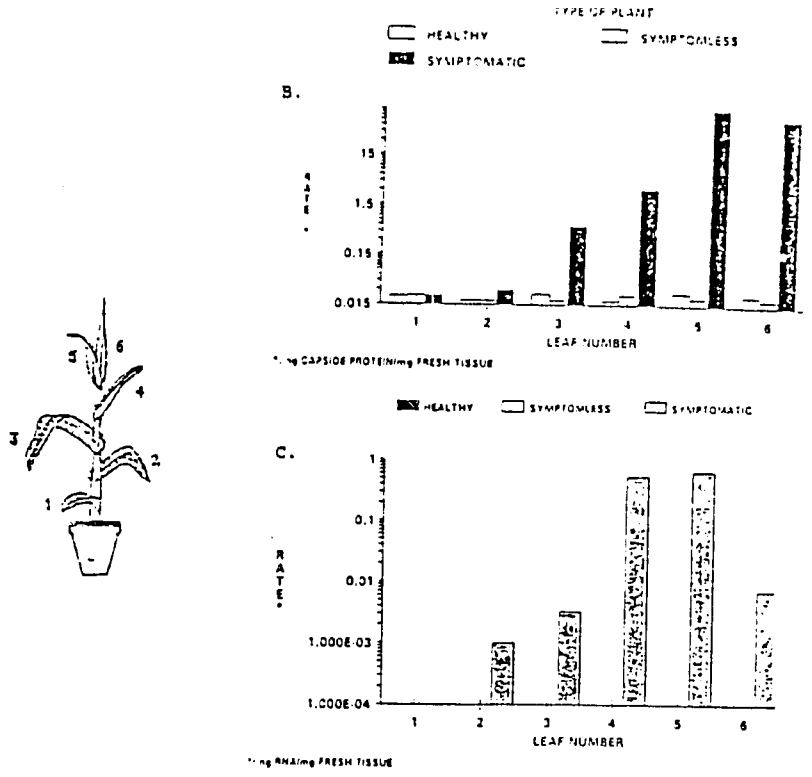


Figure 2 Analysis of the relationship between symptom expression and the accumulation of virus in maize plants infected with MRFV. A. Pattern of symptom expression in MRFV-infected plants twelve days after inoculation. Leaves 1 (inoculated leaf) - 3 were symptomless, leaves 4 - 6 exhibited typical MRFV symptoms. B. Frequency distribution of MRFV coat protein in leaves 1 - 6 as analyzed by ELISA - (a) healthy plants, (b) plants exposed to MRFV but not expressing symptoms and (c) plants exposed to MRFV and expressing symptoms (infected). C. Frequency of distribution of MRFV-RNA in leaves 1 - 6 as analyzed by slot-blot nucleic acid hybridization. Results of analysis of the same plants examined in 1B are shown - only those leaves from plants infected and expressing symptoms showed detectable levels of viral RNA.

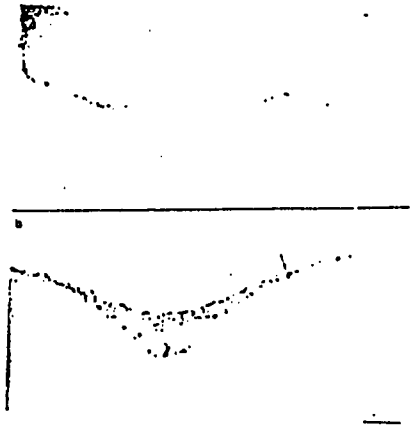


Figure 3 Tissue print of healthy (a) and MRFV-infected (b) leaf tissue as examined by nucleic acid hybridization with digoxigenin-labeled RNA probes. The arrow in (b) indicates a region of hybridization. Most MRFV-positive cells appear to be near the vascular tissue.

group containing 18 genotypes was susceptible to MRFV producing severe symptoms and a second group consisting of two genotypes from CIMMYT were tolerant to MRFV infection, ie. the virus was able to replicate to some level yet the appearance of symptoms was delayed and the level of symptom expression was reduced (Figure 4). In addition, scanning electron microscopy of select tolerant and susceptible genotypes after infection with MRFV revealed striking morphological aberrations in symptomatic leaves of both tolerant and susceptible genotypes (Figure 5) [see Bustamante et al., Appendix II].

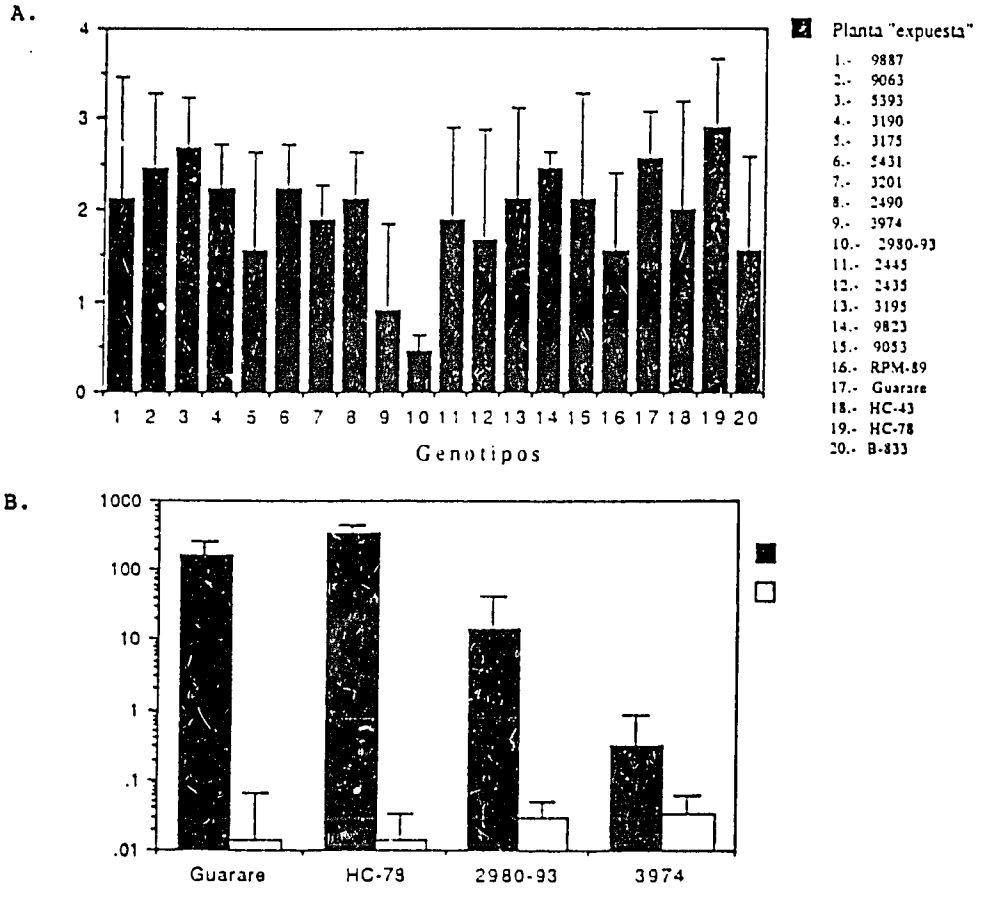


Figure 4 Evaluation of maize germplasm for resistance to MRFV. A. 500 plants composed of 20 genotypes of maize were exposed to viruliferous MRFV insects and symptoms were analyzed 15 - 18 days later. Symptom ratings were based upon a four-point scale where 0 = no symptoms and 4 = continouous lines in 75 - 100% of the leaf area with necrosis on the leaf edges. Genotype 17 is Guarare, the susceptible genotype used routinely in the lab for studies on MRFV. B. Determination of capsid protein in susceptible (Guarare, HC-78) and "tolerant" (2980-93, 3974) healthy and infected maize plants.

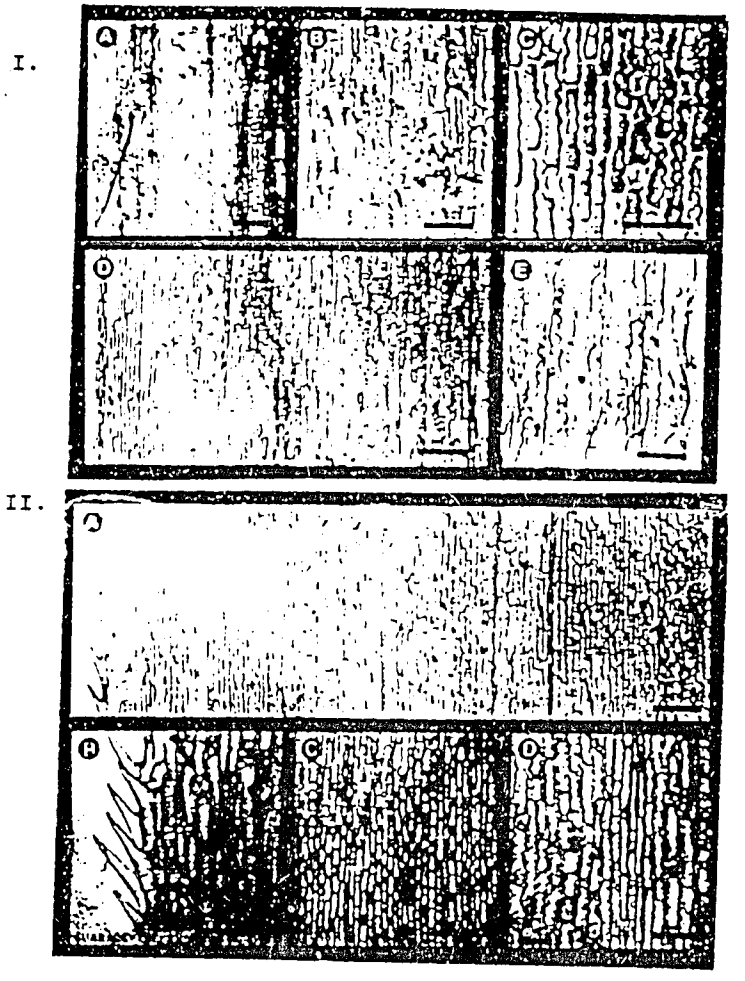


Figure 5 Scanning electron microscopy of leaves obtained from tolerant (2980-93) (I) and susceptible (Guarare) (II) genotypes of maize. Leaf tissue was taken from healthy and MRFV-infected maize plants at a time corresponding to 18 days post-inoculation. In symptomatic plants of both genotypes, the size of the stomates was reduced (see I.E and II.D). In addition, the leaf edge appeared to be more serated and disorganized (II.B). In symptomatic plants of only the susceptible genotypes, the stomates were collapsed (II.D). I. A - C, healthy; D - E, infected. II. A, Healthy; B - D, infected.

A survey of of the incidence and geographic distribution of MRFV was made in 8 Latin American countries during August - December of 1992. Maize with MRFV symptoms were found in all countries and life zones sampled. All of the collected plants were tested for the presence of MRFV using hybridization with and MRFV cRNA probe. Different patterns of MRFV symptomatology were observed and the presence of the virus was confirmed by Hybridizatoin analysis [see Kogel et al., submitted to Plant Disease, 1995]. The follow-up analysis of these samples which is not yet complete was the use of PCR and primers specific to the coat protein gene to obtain this gene from a number of the isolates representing each country and different geographic zones within and between the countries.

Restriction fragment length polymorphism (Figure 6) and sequence analysis of the PCR products has revealed interesting relationships between the isolates. The data analysis is not yet complete.

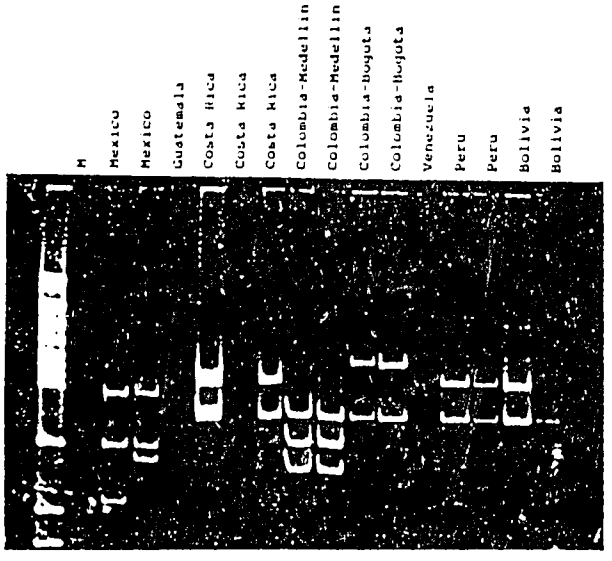


Figure 6 Restriction analysis of PCR products obtained from isolates of MRFV collected in geographically distinct regions. PCR products were obtained by using primers corresponding to a region in the 3' half of the genome, were digested with HaeIII, and the products were separated by electrophoresis through a 12% polyacrylamide gel. The marker (M) is a HaeIII digest of a pUC9 construct that is used routinely in the laboratory. From left to right, the PCR products represent samples taken from geographically distinct regions of the same country and different countries in Central and South America. The profiles exhibited in this digest as well as other restriction digests of the same products are being analyzed using the mathematical model of Nei and Li (1979) to determine the genetic relatedness of these isolates.

Objective 3. The use of molecular probes to study viral replication and accumulation in the insect host.

One area which has essentially remained untouched has been the study of the replication and accumulation of the virus in the insect vector. This part of the project has been technically challenging, necessitating additional training not available in either laboratory. BOSTID funded training in immunogold labeling and electron microscopy at Mississippi State University for a UCR graduate student (Federico Albertazzi). The experiments outlined in the proposal were not completed as the student left to pursue his doctoral thesis in Germany.

Section III. Impact, Relevance, and Technology Transfer

A. Scientific Impact of Collaboration

The following travel and training was completed during the period covered by this report:

Scientist	Travel To:	Period:	Training:
Federico Albertazzi UCR	Mississippi State University	October 1991	electron microscopy
"	USDA/ARS	November 1991	PCR
Dr. Pilar Ramirez	USDA/ARS	April - May 1992	PCR, sequencing
Dr. Pilar Ramirez	USDA/ARS	October 1992	sequencing

During the October 1992 visit, Dr. Ramirez attended and presented research results at a meeting being held October 19 - 21, 1992 at the University of Maryland entitled "Fifth International Symposium on Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance". Two posters were presented with the following titles: 1) The Pattern and Accumulation of Maize Rayado Fino Virus Capsid Proteins and RNA in Infected Maize Plants and 2) Study of the Responses of the Infection in Susceptible and Tolerant Cultivars of Zea mays L. to Maize Rayado Fino Virus.

Richard Kogel	USDA/ARS	May - July 1993	Characterization of maize samples collected in Latin America

The P. I. and U. S. Cooperator have worked together very closely in designing and performing experiments towards achieving the objectives outlined in the proposal. An LDC student, Federico Albertazzi, visited the US laboratory in November/December 1991 to obtain experience in the use of digoxigenin-labeled probes for virus detection, as well as obtaining specific training at Mississippi State University in electron microscopy and immunogold labeling. The latter training was arranged and financed by BOSTID. In April/May 1992, the P. I. visited the U.S. laboratory to perform experiments leading to the identification of the MRFV capsid protein gene. During the course of this visit, a seminar entitled"Maize Rayado Fino Virus - A Disease of Tropical and Subtropical Maize" was presented by the Drs. Ramirez and Hammond to staff of AID at the Rosslyn, Va. AID/PSTC office. In addition, Dr. Ramirez the PCR technology that will be used by one her students to evaluate strain diversity of MRFV isolates to be collected throughout

Central and South America in September/December 1992. The US cooperator has continued with cDNA cloning, sequencing, and development of new technologies to be transferred to the LDC laboratory. She also visited the LDC laboratory in September 1992 in order to confer with the P. I. on the annual report, on progress made to date, and on the design of future experiments.

B. Description of Project Impact

The results of the studies performed by Federico Albertazzi on the pattern and accumulation of MRFV in infected maize plants laid the groundwork for the subsequent study by Pedro Bustamante on the response of tolerant and susceptible genotypes of maize to MRFV infection. During the course of the latter study, we have developed the technology to index maize breeding lines and germplasm collections for tolerance to MRFV. The MRFV coat protein gene identified during the course of this research will be used to develop coat protein-mediated protection in transgenic maize plants. The results obtained thus far from this project will be very useful in introducing resistance to MRFV into maize cultivars grown by farmers, as well as providing more basic information about the interaction of MRFV with its maize host.

C. Technology Transfer

Important progress has been made in the strengthening of research capabilities at this Institution through the purchasing of equipment and reagents and the development of research skills. In addition to the purchase of a refrigerated incubator for storage of materials, reagents such as non-radioactive nucleic acid detection kits, reagents for extraction and electrophoresis of proteins and nucleic acids, as well as primers and reagents for polymerase chain reaction (PCR) analysis of MRFV isolates have been purchased. The P.I. and graduate students from Costa Rica have been trained in cDNA cloning, nucleic acid sequencing, nucleic acid hybridization, tissue printing, PCR technology, and the design of experiments and analysis of results. The University of Costa Rica has developed BITNET facilities which have been used to establish an efficient scientific linkage with the US.

Significant difficulties were encountered in the management of the project (See Appendix I). As a result of these difficulties and in an effort to overcome institutional constraints, Dr. Ramirez is promoting a new system of administration for her Institute in order to have better control over the expenditure of grant funds without hampering the progress of this and future projects. In Costa Rica, one of the most severe constraints of research progress is the inconsistent system for the import of perishable materials. In addition, the inflexibility of the AID mission in the procurement of materials has led to delays which have hindered performance of the research. The last two items have been partially overcome by a significant effort on the part of the US collaborator in the purchasing and delivery of reagents and perishable materials to her Costa Rican collaborator.

Section IV. Project Activities/Outputs

- The following poster was presented at the **American Phytopathology Society** meeting , 1990:

Ramirez, P., Karakashian, J., de los Angeles Mora, M., and Hammond, R.W. Dot blot hybridizatoin assay for detection of maize rayado fino virs (MRFV) in plant extracts using biotinylated probes (Phytopathology 80: 516, 1990.)

- The PI and co-PI both attended and presented at the **Vth International Symposium on Biotechnology and Plant Protection : Viral Pathogenesis and Disease Resistance** at the University of Maryland, College Park, MD October 1992. Two posters were presented entitled:

Albertazzi, F., Hammond, R. W., Rivera, C., and Ramirez, P. The pattern of accumulation of maize rayado fino virus capsid proteins and RNA in ifnected maize plants.

Bustamante, P., Albertazzi, F., Hammond, R., and Ramirez, P. Study of the responses of the infection in susceptible and tolerant cultivars of **Zea mays** L. to maize rayado fino virus.

- A poster was also presented at the **IXth International Congress of Virology**, Glasgow, Scotland August, 1993, entitled:

Bustamante, P., Albertazzi, F., Hammond, R., and Ramirez, P. 1993. Resistance in **Zea mays** L. against maize rayado fino virus.

One manuscript has been published and several manuscripts are in preparation or are submitted to peer-reviewed journals. They include the following and are included in Appendix II, in the following order:

1. Ramirez, P., Hammond, R. W., Karkashian, J., and Mora, M. 1990. Production of biotinylated nucleic acid probes for detection of maize rayado fino virus. Revist de Invest. Agr. 22: 50-55.

2. Albertazzi, F., Ramirez, P., Hammond, R. W., and Rivera, C. The pattern of accumulation of MRFV in infected maize plants. (in preparation for submission to Phytopathology).

3. Kogel, R., Hammond, R. W., and Ramirez, P. Incidence and geographic distribution of maize rayado fino virus (MRFV) in Latin America identified by dot-blot hybridization.

(submittted to Plant Disease).

4. Bustamante, P. I., Hammond, R., and Ramirez, P. Evaluation of maize germplasm for resistance to maize rayado fino virus (in preparation for submission to Phytopathology)

Section V. Project Productivity

The project accomplished more than was expected at the time the proposal was written. We were able to identify the coat protein gene as was explained previously. Maize germplasm lines exhibiting tolernace/resistance to the virus were identifed. The geographic distribution of MRFV and strain diversity have been examined - information valuable to determining factors which influence the epidemiology of the disease. The nucleotide sequence of the geogprahically diverse isolates is useful in order to design better constructions for engineering MRFV-resistant maize.

Section VI. Future Work

Follow-up experiments were proposed in an AID/PSTC full proposal (pre-proposal attached in Appendix III); unfortunately, although our proposal was considered by the peer-review panel to be worthy of funding, the PSTC program was discontinued that year. We are continuing the project on our own while seeking funding from alternative sources. The full-length sequence of MRFV will be completed this year, and analysis of strain diversity will also be completed this year. Experiments designed to examine the expression of the virus in maize and the insect host await further funding. Our collaboration with other groups for the production of virus-resistant, transgenic maize containing the MRFV coat protein is continuing. We are continuing to evaluate tolerant and susceptible germplasm from CIMMYT. Selected tolerant and susceptible germplasm will be chosen from the evaluation to perform more comprehensive studies on replication and movement of the virus in order to understand the mechanisms involved in tolerance.

Section VII. Literature Cited

Gamez, R. 1969. A new leafhopper-borne virus of corn in Central America. Plant Disease Reporter 53, 929-932.

Gamez, R., and Leon, P. Maize rayado fino virus and related viruses. In: The Plant Viruses. (R. Koenig, Ed.) Plenum Press, New York, pp. 213-223.

Leon, P., and Gamez, R. 1981. Some physicochemical properties of maize rayado fino virus. J. gen. Virol. 56, 67-75.

Nei, M., and Ki, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. natl. Acad. Sci. USA 76, 5269-5273.

Ramirez, P., Hammond, R., Karkashian, J., and Mora, M. A. 1990. Production of biotinylated probes for detection of maize rayado fino virus. Revista de Investigaciones Agricolas RIA. 12, 50-55.

APPENDICES

APPENDIX I

Managerial Issues

Since the time that the grant was awarded to the University of Costa Rica, on June 30, 1989, many issues have arisen as to allocation and spending of funds. An agreement could not be reached between the AID mission, the UCR and USDA as to how the funds to be spent by USDA were to be managed. In the end, a PASA between USDA/OICD and AID was arranged and funding to the USDA/ARS laboratory began in August, 1991.

Similar problems were encountered by the LDC laboratory in trying to obtain and spend the funds held by the AID Mission. The funding at the LDC laboratory began in March, 1991. The difficulties in procurement of materials have remained a constant barrier towards research progress in the LDC labortory. Many of the problems encountered may have been due to inexperience of the Project Officer assigned to our project at its outset.

Difficulties were also encountered in the management of AID funds transferred to the University. In July of 1992, the LDC scientist observed irregularities in the financial reports concerning the expenditure of her grant funds. Subsequent investigation by University authorities and the AID mission revealed that certain funds had been stolen by a University accountant entrusted with their disbursement. Legal action was pursued by the University.

As the grant was awarded June 30, 1989 for a three year period, and as we have only just begun receiving the funds, we asked and received a time extension of one year in order to complete the project with AID funding.

Appendix II.

Manuscripts

Manuscripts published or being prepared for submission to peer-reviewed journals are attached in the following order:

1. Ramirez, P., Hammond, R. W., Karkashian, J., and Mora, M. 1990. Production of biotinylated nucleic acid probes for detection of maize rayado fino virus. Revist de Invest. Agr. 22: 50-55.

2. Albertazzi, F., Ramirez, P., Hammond, R. W., and Rivera, C. The pattern of accumulation of MRFV in infected maize plants. (in preparation for submission to Phytopathology).

3. Kogel, R., Hammond, R. W., and Ramirez, P. Incidence and geographic distribution of maize rayado fino virus (MRFV) in Latin America identified by dot-blot hybridization. (submittted to Plant Disease).

4. Bustamante, P. I., Hammond, R., and Ramirez, P. Evaluation of maize germplasm for resistance to maize rayado fino virus (in preparation for submission to Phytopathology)

PRODUCTION OF BIOTINYLATED NUCLEIC ACID PROBES FOR DETECTION OF MAIZE RAYADO FINO VIRUS

RAMIREZ, P., HAMMOND, R.W., KARKASHIAN, J. and M. de los A. MORA.

Centro de Investigación en Biología Celular y Molecular. Universidad de Costa Rica, San José, Costa Rica. Microbiology and Plant Pathology Laboratory, Plant Science Institute, USDA-ARS Beltsville Agricultural Research Center, Beltsville, Maryland 20705, USA.

SUMMARY

A solid-phase nucleic acid hybridization technique for the detection of maize rayado fino virus (MRFV) RNA in maize tissue is described. Total nucleic acids from infected plants were extracted by phenol-chloroform treatment and precipitated with ethanol before spotting. A cloned cDNA labelled with biotin specifically recognized picogram quantities of viral RNA in maize total nucleic acids under high stringency hybridization conditions. Cellular nucleic acids did not interfere with cDNA-target viral RNA hybridization, and denaturation of the sample before spotting increased sensitivity by up to 16-fold compared to non-denatured samples. This technique may be used as a routine procedure for the indexing of Maize Rayado Fino disease.

RESUMEN

En este trabajo se describe el desarrollo de una técnica de hibridización de ácidos nucleicos en fase sólida, para la detección de RNA del maize rayado fino virus (MRFV) en tejidos de maíz. Mediante un tratamiento con fenol-cloroformo se extrajo la totalidad de los ácidos nucleicos de las plantas infectadas y se los precipitó con etanol antes de ser sembrados. Bajo condiciones de hibridización muy estrictas, un cDNA clonado marcado con biotina reconoció en forma específica cantidades del orden de los picogramos de RNA viral, del conjunto de ácidos nucleicos totales del maíz. Los ácidos nucleicos celulares no interfirieron en la hibridización del RNA con su respectivo cDNA, y la desnaturalización de la muestra antes de la siembra aumentó la sensibilidad hasta 16 veces. Esta técnica puede ser utilizada como procedimiento de rutina para el indexing de la enfermedad causada por el maize rayado fino virus.

Keywords: Virus, MRFV, indexing, nucleic-acid, probes.

The leafhopper-born Maize Rayado Fino Virus (MRFV) is the only known indigenous virus of maize in Central America (Gámez and León, 1983).MRFV is the best characterized member of the Marafivirus group of plant viruses, which includes oat blue dwarf virus (OBDV) and bermuda grass etched line virus (BELV) (Gámez and León, 1988). MRFV consists of small isometric particles, ca. 30 nm in diameter and a single-stranded RNA genome with M_r of 2.0-2.1 x 10^6 (León and Gámez, 1981). One characteristic

of the virus is its ability to replicate in both maize and its insect vector, *Dalbulus maidis* (Rivera and Gámez, 1986). Under widely dissimilar environments, MRFV and *D. maidis* may cause epidemics (Gámez and Saavedra, 1986). Although no resistant maize cultivars are known, they differ in their susceptibility to MRFV (Toler *et al.*, 1985).

Symptoms of MRFV infection include the presence of distinct small chlorotic dots which develop at the base and along the veins of young leaves and some of these coalesce to form short stripes. The phenotypic expression of symptoms depends upon the genetic constitution of both the host plant and the virus and is characteristic of a particular combination of both. With many viral diseases, including MRFV, the degree of symptom severity is not always proportional to viral replication within the host. In addition, MRFV most often occurs in field infections associated with mollicutes (corn stunt spiroplasma and maize bushy stunt mycoplasma) which are transmitted by the same vector (Gámez and León, 1985). Under certain environmental conditions, the symptoms of MRFV infection may be masked by symptoms produced by infection with the mollicutes.

We have developed a nonradioactive detection method for MRFV using nucleic acid spot hybridization. This method will be extremely useful as a means of diagnosis of the virus in field infections, and as a means of indexing breeding lines and germplasm collections for tolerance to MRFV.

Maize seeds were germinated on moistened dishes for 3 days and then planted and grown under greenhouse conditions. Virus inoculation was as described by Gámez (1973) and plants were extracted two weeks after inoculation, when symptoms developed. Tissue sample preparation was by a modification of the procedures described by Palukaitis *et al.* (1985) and Laulhere and Rozier (1976). Briefly, inoculated or healthy maize plants were selected randomly, and 0.3g of leaf tissue were ground with sterile sand and 0.5ml of AMESS buffer (0.5M NaOAc, 10mM $MgCl_2$, 20% ethanol, 3% SDS, 1M NaCl, pH 6.0), in a microfuge tube and incubated at 37°C for 5 minutes. Tissue debris was removed by brief centrifugation. The clarified sap was extracted with 1/2 volume of water-saturated phenol and 1/2 volume of chloroform-isoamyl alcohol (24:1). After a brief centrifugation, the aqueous layer was reextracted with one volume of chloroform-isoamyl alcohol and total nucleic acids were precipitated with 2.5 volumes of ethanol and 1/20 volume of 3M NaOAc. The precipitate was resuspended in 20 μl of cold sterile twice-distilled water. After clarification, an aliquot was diluted 1:200 with water and the absorbance at 260nm was measured to estimate the amount of total nucleic acids present. Processing of 0.3g of leaf tissue generally yielded about 10μg of total nucleic acids which were diluted and used for blotting.

Serial dilutions of samples were prepared with 6x SSC (native conditions) or with 6x SSC / 7.5% formaldehyde (denaturing conditions). Samples treated with formaldehyde were incubated at 60°C for 10 minutes and rapidly ice-cooled, just prior to spotting. Aliquots of 150μl were applied with a Bio-Dot apparatus (BioRad, Richmond, CA) to 0.45μm pore nitrocellulose

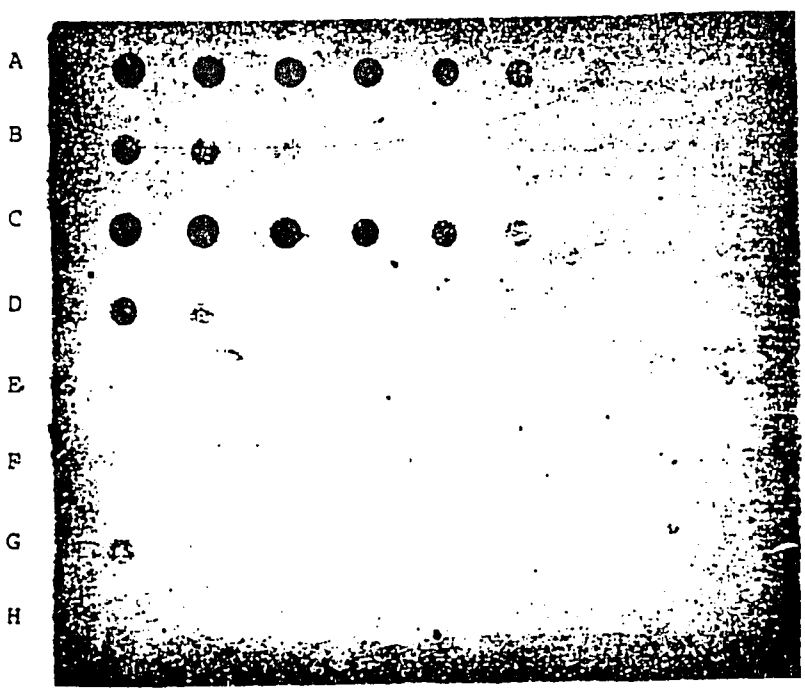
membranes (Schleicher and Schuell, Dassel, West Germany) which had been air-dried following presoaking in 20x SSC. After sample application, the membranes were baked at 80°C for 2 hours.

Filters were prehybridized in 5x SSC, 5x Denhardt's solution with 0.4% bovine serum albumin, 150μg/ml heat-denatured salmon sperm DNA, 50mM sodium phosphate pH 6.5, 0.1% SDS and 50% formamide, for 2 hours at 50°C. Hybridization was carried out overnight at 50°C, in the same solution containing 150ng/ml of biotinylated probe. The probe was a plasmid containing a cloned, 800bp cDNA of MRFV RNA. The cDNA was prepared by polyadenylation of the viral RNA at it's 3'-OH terminus, followed by annealing to oligo-dT in a buffer containing 5.0mM $NaPO_4$ pH 7.2, 2.0mM EDTA and 15% Ultrapure methyl sulfoxide-d6 (Aldrich, Milwaukee, WI), according to Polites and Marotti (1986). This primed RNA was used as a template for retrotransciption with a cloned Moloney murine leukemia virus reverse transcriptase (M-MLV RT from BRL, Gaithersburg, MD)(D'Alessio *et al.*, 1987). The second strand cDNA was produced in a reaction catalyzed by the large fragment of *E. coli* DNA polymerase I (Klenow fragment)(Sanger *et al.*, 1977). The double stranded DNA was then inserted into pUC9 at the PstI site by the "dG-dC tailing" method (Efstratiadis and Villa-Komaroff, 1979). Labelling of the probe was accomplished by nick translation with a commercial kit using biotin-11-dUTP (BRL, Gaithersburg, MD). Visualization of spots was made with the BRL DNA Detection System, using streptavidin and a biotin-conjugated alkaline phosphatase.

In preliminary experiments, hybridizations were done under low stringency conditions. In 6x SSC / 45% formamide at 42°C, samples of healthy plant nucleic acids gave positive reactions with approximately the same intensity as samples from infected plants. Higher stringency conditions were tested and controls were added to determine the nature of any interference of plant nucleic acids with cDNA / viral RNA hybridization. These experiments resulted in a sensitive and specific recognition of viral RNA by the biotin-labelled cDNA probe. An almost identical pattern in spot intensities is found when lanes A and B (MRFV-RNA) are compared with lanes C and D (MRFV-RNA + healthy plant nucleic acids)(Fig. 1). Moreover, the samples derived from healthy plants did not show any hybridization to the probe (Fig. 1, lanes E and F). This suggests that plant nucleic acids caused no significant interference with cDNA / target RNA hybridization. When this cDNA was assayed for hybridization with a negative control (tobacco mosaic virus genomic RNA), the probe failed to hybridize even under low stringency conditions (data not shown). Initial dot-blot hybridization experiments using the biotin labelled probe indicated that crude sap as well as undiluted nucleic acid extracts prepared from healthy maize tissue gave false positive signals on nitrocellulose. This problem was overcome by the phenol-chloroform extraction procedures, ethanol precipitation and dilution of samples, making the first extract spotted equivalent to 1μg of total nucleic acids.

Denaturation with 7.5% formaldehyde of MRFV genomic RNA, clearly increased the sensitivity of the detection by up to 16-fold compared to non-denatured RNA (Fig.1, lanes A and C compared with B and D; and results not shown). Denaturation of total nucleic acids from infected maize tissue increased the sensitivity of detection by up to 4-fold (Fig.1, lane G compared to lane H). This is in agreement with the results obtained by Habili *et al.* (1987) with Barley Yellow Dwarf Virus (BYDV). The limit of detection of our technique was 100 - 120 pg of viral RNA. In the blot presented in figure 1, this represents a ratio of about 1:1000 between viral RNA and total nucleic acids.

Fig. 1. Detection of MRFV-RNA in leaf extracts of infected maize plants by nucleic acid spot hybridization. Lanes A and B: Two-fold dilutions of MRFV genomic RNA (30ng to 120pg). Lanes C and D: same as A and B but combined with two-fold dilutions of total nucleic acids from a healthy plant (1μg to 4ng). Lanes E and F: Two-fold dilutions of total nucleic acids from a healthy plant (1μg to 4ng). Lanes G and H: Two-fold dilutions of total nucleic acids from an infected plant (1μg to 4ng). Lanes A,C,E and G contain denatured nucleic acid samples. Lanes B,D,F and H contain native nucleic acid samples.



22'

Denaturation of RNA has been stated to be unnecessary for dot-blotting. Maule *et al.* (1983) reviewed the effect of several denaturing agents on the sensitivity of detection for cowpea mosaic virus (CPMV), pea enation mosaic virus (PEMV), alfalfa mosaic virus (AlMV) and tobacco mosaic virus (TMV). These included the use of glyoxal, NaOH, and formaldehyde on virus preparations and tissue homogenates. Our results using 7.5% formaldehyde both with the MRFV genomic RNA and with total nucleic acids from infected maize tissue, show that this treatment increases sensitivity of the technique by up to 16-fold. The increase in sensitivity may reflect maximum binding to nitrocellulose due to a quantitative denaturation of the RNA. It is also possible that MRFV-RNA contains considerable secondary structure that could interfere with hybridization.

The use of nucleic acid hybridization to detect this virus could be a useful alternative to ELISA and symptom production. Biotin labelling of the probe provides several advantages for underdeveloped countries, including reduced hazards, lowered costs and longer probe shelf life.

Acknowledgments

We wish to acknowledge the technical help of Reynaldo Pereira with photografic material.

REFERENCES

D'ALESSIO, J.M., NOON, M.C., LEY, H.L. and G.F. GERARD. 1987. One-tube double-stranded cDNA synthesis using cloned M-MLV reverse transcriptase. Focus 9(1): 1-8.

EFSTRATIADIS, A. and L. VILLA-KOMAROFF. 1979. Cloning of double-stranded DNA. In: Genetic Engineering. Edited by J.K. Setlow and A. Hollaender. Plenum Press, New York. 1: 15.

GAMEZ, R. 1973. Transmission of rayado fino virus of maize (Zea mays) by Dalbulus maidis. Ann. Appl. Biol. 73: 285-292.

GAMEZ, R and P. LEON. 1983. Maize Rayado Fino Virus: Evolution with plant host and insect vector. In: Current Topics in Vector Research. Edited by K.F. Harris. Praeger Publishers, New York. 1: 149-168.

GAMEZ, R. and P. LEON. 1985. Ecology and evolution of neotropical leafhopper-virus-maize association. In: The Leafhoppers and Planthoppers. Edited by L.R. Nault and J.G. Rodriguez. J. Wiley and Sons, Inc., New York. pp. 331-350.

GAMEZ, R. and P. LEON. 1988. Maize Rayado Fino and Related Viruses. In: The Plant Viruses. Edited by R. Koenig. Plenum Publishing Corporation, New York. 3: 213-233.

GAMEZ, R. and R. SAAVEDRA. 1986. Maize Rayado Fino: A model of a leafhopper-borne virus disease in the Neotropics. In: Plant Virus Epidemics. Edited by G. MacLean, R. Garret and W. Ruesink. Academic Press, Melbourne. pp. 315-325.

HABILI, N., McINNES, J.L. and R.H. SYMONS. 1987. Nonradioactive, photobiotin-labelled DNA

probes for the routine diagnosis of Barley Yellow Dwarf Virus. Journal of Virological Methods 16: 225-237.

LAULHERE, J.P. and C. ROZIER. 1976. One-step extraction of plant nucleic acids. Plant Science Letters 6: 237-242.

LEON, P. and R. GAMEZ. 1981. Some physicochemical properties of Maize Rayado Fino Virus. Journal of General Virology 56: 67-75.

MAULE, A.J., HULL, R. and J. DONSON. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. Journal of Virological Methods 6: 215-224.

PALUKAITIS, P., COTTS, S. and M. ZAITLIN. 1985. Detection and Identification of Viroids and Viral Nucleic Acids by Dot-Blot Hybridization. Acta Horticulturae 164: 109-118.

POLITES, H.G. and K.R. MAROTTI. 1986. A step-wise protocol for cDNA synthesis. Biotechniques 4(6): 514-520.

RIVERA, C. and R. GAMEZ. 1986. Multiplication of Maize Rayado Fino Virus in its leafhopper vector Dalbulus maidis. Intervirology 25: 76-82.

SANGER, F., NICKLEN, S. and A.R. COULSON. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences U.S.A. 74: 5463-5467.

TOLER, R.W., SKINNER, G. and A.J. BOCKHOLT. 1985. Reactions of maize (Zea mays) accessions to Maize Rayado Fino Virus. Plant Disease 69(1): 56-57.

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The Pattern of Accumulation of MRFV in Infected Maize Plants

F. Albertazzi[1], P. Ramírez[1,2], R.W. Hammond[3] and C. Rivera[1,4]

[1] Centro de Investigacion en Biología Celular y Molecular, Universidad de Costa Rica, San José, Costa Rica.

[2] Escuela de Biología, Universidad de Costa Rica.

[3] USDA-Agricultural Research Service, Molecular Plant Pathology Laboratory, Beltsville, Maryland, U.S.A.

[4] Escuela de Microbiología, Universidad de Costa Rica

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ABSTRACT

The leafhopper-borne maize rayado fino virus (MRFV) is transmitted in a persistent manner to maize in which severe disease symptoms can occur. The concentrations and distribution of MRFV viral RNA and coat protein have been examined in relation to symptom expression in infected maize plants. The results show that symptom severity was directly correlated with the accumulation of coat protein and viral RNA. Younger leaves accumulated more viral products than older leaves and there was a progressive basipetal (leaf tip to base) decline in the amount of coat protein in the infected leaves. Coat protein and viral RNA were detected in asymptomatic as well as symptomatic leaves but not in the inoculated leaf. Both coat protein and viral RNA could be detected in stem and secondary roots by tissue prints, but no viral RNA was detected in roots by slot-blot hybridization. In situ hybridization of maize leaves shows the accumulation of MRFV RNA in the vascular bundles. The results confirm that viral distribution is affected by the plant growth and provide a picture of the relationship between some viral products of MRFV and leaf development.

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Maize rayado fino virus (MRFV) is the type member of the Marafivirus group of plant viruses. Symptoms of MRFV infection in maize consist of chlorotic dots at the base and along the veins of the leaves, which become increasingly numerous in subsequent new leaves and occasionally coalesce to form stripes. The geographical distribution of MRFV is neotropical, extending from the southern United States to northern Argentina and Uruguay (Gámez & León,1987). MRFV is transmitted in a circulative-propagative manner by the cicadellid leafhopper Dalbulus maidis de Long & Wolcott. MRFV has a genome consisting of one RNA molecule of mol. wt. approx. 2 millon. The coat protein has a pH range of 6 to 6.2 and a mol. wt of 20 kDa. (León & Gámez,1981).

Most knowledge of virus-host interactions derives from genetic and molecular studies of the hypersensitive response (Tobias et al.,1989). However, with the exceptions of cauliflower mosaic virus (CaMV) (Covey et al.,1990; Leisner et al.,1992) and tobacco mosaic virus (TMV) (Wisniewsky et al.,1990), relatively little is know at molecular level about virus-host interactions in the systemic response to infections. Studies related to the pattern of accumulation and local and systemic dispersion are fundamental to understanding how viruses infect their host.

In this paper we report the pattern of accumulation of MRFV viral RNA and coat protein in infected maize plants. We employ the techniques of DAS-ELISA, slot blot hybridization, tissue printing, and in situ hybridization, and use monoclonal and polyclonal MRFV-coat protein antisera and MRFV riboprobes to monitor virus accumulation.

The MRFV-CR (Costa Rican) isolate was maintained in the susceptible Zea mays L.

var. Guararé as described by Gámez (1972). Approximately 150 third or fourth instar leafhoppers were placed for 3 days on infected plants to acquire the virus. The insects were transferred every 3 days to healthy plants for a period of 18 days to complete the incubation phase of the virus in the insects. The leafhoppers were randomly divided into groups of 5-8 insects each and placed on maize plants (having three leaves) on the middle of the first leaf in a tube cage for a total of 10 plants per inoculation. These insects were transferred at a rate of 5 to 8 insects per healthy test plant in series of two to four consecutive, 3- to 4 day-long inoculation periods. The plants were sprayed with systemic insecticide and placed in a greenhouse for symptom expression. Plants that were exposed to non-viruliferous leafhoppers (healthy insects) are referred to as "healthy" and served as controls. Plants that were exposed to viruliferous insects but never developed symptoms are referred to as "exposed" and those which expressed symptoms as are referred to as "infected". The experiment was repeated twice. The acquisition, incubation, and inoculation were performed in a growth chamber with a photoperiod of 16 hr and temperature range of 18-28°C.

Tissue samples were taken from the roots and the distal and proximal parts fo the leaves of healthy, exposed, and MRFV-infected plants. 10 to 25 mg of fresh tissue was ground in 1 ml of 0.015 M phosphate-buffered saline containing 4% BSA and 0.05% Tween-20 (PBSAT). Doublew-antibody sandwich (DAS) ELISA was performed as described by Clark & Adams (1977) with minor modifications, using specific polyclonal antibodies to the MRFV-coat protein and Polystyrene microtitre plates (Dynatech Corp., Alexandria, VA) previously coated with IgG at a concentration of 2 ug/ml in 0.05M in sodium carbonate buffer, pH 9.6. After incubation for 2 days at 4°C, the wells in the plates were washed with 200 μl of 0.015 M phosphate-buffered saline containing 0.05% Tween-20 (PBST), the samples were added to the plates which were

further incubated overnight at 4°C. Alkaline phosphatase-labeled IgG conjugate ( ) was added to the wells, previously washed with PBST, and the plates were incubated 2 hr at 37°C. Finally, 200 µl of BCIP/NBT? substrate (Sigma, St. Louis, MO, U.S.A.) was added to each well at a concentration of 0.6 mg/l in 10% diethalonamine in 0.015 M phosphate-buffered saline, pH 9.8. The resulting color reactions were assessed spectrophotometrically (405 nm) after 2,5,15,30,60 and 120 min. Values higher than the negative control plus three standard deviations of the negative control mean were considered positive.

MRFV-RNA was obtained by phenol-isoamyl extractions from purified MRFV according to León & Gámez (1981). Total RNA from 10 to 20 mg of fresh root tissue and the proximal section of the leaves of healthy, exposed and MRFV-infected plants were extracted as described by Verwoerd et al.(1989). Dilutions of denatured MRFV-RNA and total RNA were concentrated on nitrocellulose sheets (Schleicher & Schuell, Germany) in a Slot-Blot apparatus (Bio-Rad, Richmond, Ca,U.S.A.) at 200 µl/slot.

A cDNA clone of MRFV-CR (Ramírez & Hammond, 1987) was used as template to synthesize RNA transcripts labeled with digoxigenin (DIG) using the Boehringer RNA Labeling Kit SP6/T7 (Boehringer Mannheim, Germany) according manufacturers instructions. The filters with RNA were UV-cross-linked, prehybridized, hybridized and washed according to the protocols of the Boehringer Mannheim Nucleic Acid Detection Kit (Boehringer Mannheim, Germany). The filters were developed with Lumi-Phos™ 530 (Boehringer Mannheim, Mannheim, Germany) according to manufacturers instructions and were exposed to Hyperfilm ECL (Amersham, Buckinghamshire, England).

Tissue print immunoblots and tissue print RNA blots were performed as described by Ye and Varner (1991). All prints of leaves, stems and secondary roots were done by free-hand

as cross- or longitudinal- sections. After printing, the membranes were dried and probed with monoclonal antibodies or RNA-probes. For the MRFV-coat protein detection, three monoclonal antibodies were used (León et al., in preparation). The RNA-probes hybridizes with complementary RNA pertaining to coat protein gene.

In situ hybridization of leaf cross-sections was performed essentially as described by Cox & Goldberg (1988), using a ^{35}S-labeled RNA probe complementary to the viral RNA as a probe.

Infected plants at 30 days of age expressed symptoms 7-12 days after the transmission period. Leaves 1,2, and 3 were asymptomatic, the proximal section of leaf 4 showed mild symptoms, and leaves 5 and 6 showed severe symptoms (Fig.1). All leaves except the leaf 1 were found to contain viral coat protein by DAS-ELISA (Fig.2). The level of MRFV coat protein was found to be higher in the proximal as compared to the distal section of leaves of infected plants, and this difference was significant at P=0.05 as determined by the t-test (Table 1). MRFV coat protein was detected in the secondary roots of infected plants. (Fig. 3). Leaves of exposed and healthy plants scored negative by DAS-ELISA. The replicate experiments were not significantly different as determined by the t-test at P=0.01.

MRFV viral RNA was detected in leaves 2,3,4,5 and 6 in infected plants. The amount of viral RNA was abundant in leaves 4 and 5 but decreased in leaf 6 (Fig. 4). This pattern of RNA accumulation was similar to the coat protein measured by DAS-ELISA. Viral RNA was not detected in secondary roots, although the presence of very small amounts of MRFV RNAs cannot be totally excluded by this detection method.

The tissue immunoblot results showed that MRFV coat protein was present in leaves 4 through 6 in infected plants. The coat protein was scattered in leaves, roots and stem in a pattern corresponding to vascular tissues and epidermis (Fig. 5). The MRFV viral RNA was

detected in the epidermal and vascular tissues of leaves 4 through 6, in secondary roots, and in the stem of infected plants (Fig. 6).

Symptom development may be a consequence of an interaction between virus-encoded proteins and host constituents (van Loon et al.,1990). Consequently, we attempted to find a correlattion betweene the pattern of symptom expression and the accumulation of coat protein and viral RNA throughout the growth and expansion of infected maize leaves. A correlation between accumulation of coat protein and appearance of symptoms has previously been observed with other plant RNA viruses, for example, tobacco mosaic virus (TMV) (von Sengbusch 1965; van Telgen et al., 1985; Dawson et al.,1988); with plant DNA viruses, eg. maize streak virus (MSV) (Lazarowitz et al.,1989) and African cassava mosaic virus (ACMV) (Fargette et al.,1987). However, it is know that for TMV some types of symptoms in a specific host do not correlate with coat protein accumulation (Lindbeck et al.,1992), and the untranslated regions can affect symptom severity, such as the 3'-terminal region tobacco vein mottle virus (TVMV) (Rodríguez-Cerezo et al.,1991). We used protein and viral RNA as partial indicators of MRFV factors that could be involved with the expression of symptoms in maize. Symptom severity was directly correlated with the accumulation of coat protein and viral RNA. The patterns of RNA and coat protein accumulation were similar, which corresponds to the results obtained for CaMV by Leisner et al. (1992). In our hands, viral RNA could not be extracted from secondary roots, althrough they could be detected by tissue print hybridization. Fuentes & Hamilton (1991) could detect coat protein in roots of two strains of southern bean mosaic virus (SBMV) by ELISA but they did not find SBMV RNA in total RNA extracted from roots. However, the presence of very small amounts of MRFV-RNA cannot be totally ruled out.

The presence of symptomless and symptom-expressing leaves in the MRFV-infected plant may be explained by the parameters which govern the movement of photoassimilate (Turgeon, 1989) and maize leaf development gradients (Sharman, 1942). In our study, MRFV was most likely inoculated directly into the vascular system in the leaf 1 by the insect. It was able to move without replication and infect the young leaves and tissues or even the secondary roots (Samuel 1934). The asymptomatic leaves 2 and 3, which were already expanded at time of inoculation, import less MRFV than leaves 4 through 6. Matthews (1991) states that in monocotyledons, one or few leaves above the inoculated leaf expanded at time of inoculation show no strip pattern of pathogen disease. Leisner et al. (1992) documented that both virus (CaMV) and photoassimilates generally accumulated in younger (sink) leaves on the side leaf nearest the point of insertion of the mature inoculated (source) leaf. A similar situation may explain our results with MRFV and infection of maize.

The differential symptom expression and accumulation of coat protein and viral RNA between the proximal and distal part of the leaves might also be explained with the same model of sink-source transition. Although an important factor in our case could be the developmental gradient from base (young) to tip (old) of maize leaves. Langdale et al. (1988) reported that relative amounts of C4 mRNAs (related to photosynthesis) change throughout leaf growth and exhibit an accumulation peak in the nonexpanded leaf blade. Once the leaf blade is fully expanded, levels of C4 mRNA decrease in the leaf sheath. Another explanation could be the "molecular gates" hypothesis of Goodrick et al. (1991) between bundles sheath and vascular tissue. The "molecular gates" could be more selective in the mature expanded leaves or tissues than in the younger ones, thereby preventing MRFV from producing symptoms in these tissues. However, this concentration threshold could be overcome in different maize cultivars where

the "molecular gates" could be less restrictive or by MRFV strains which could modify these barriers.

Acknowledgments

We thank Marilyn Hale, William Villalobos, Guillermo Rodríguez, Fernando Cabezas, Rafael Marín and Gina Durán for excellent technical assistance, and Dr. Joachim Rodríguez for critical reading of the manuscript. We also thank to Reynaldo Pereira for help with the photos. This research was supported by USAID Competitive Grant 9.189.

References

Clark, M.F. & Adams, A.N. (1977) Characteristics of the microplate method of secondary immunosorbent assay for the detection of plant viruses. J.Gen. Virol. 34:475-483.

Cox, K. H. & Goldberg, R. B. (1988) Analysis of plant gene expression In: Plant Molecular Biology - A Practical Approach (Shaw, C. H., ed.) IRL Press, Oxford, England, pp. 1-35.

Dawson, W.O., Bubrick, P., & Grantham, G.L. (1988) Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78:783-789.

Fargette, D., Thouvenel, J-C., & Fauquet, C. (1987) Virus content of leaves of cassava infected with African cassava mosaic virus. Ann. Appl. Biol. 110: 65-73.

Fuentes, A.L. & Hamilton, R.I. (1991) Sunn-hemp mosaic virus facilitates cell-to-cell spread of southern bean mosaic virus in a nonpermissive host. Phytopathology 81: 1302-1305.

Gámez, R. (1973) Transmission of rayado fino of maize (Z. mays) by Dalbulus maidis. Ann. Appl. Biol. 73: 285-292.

Gámez, R. & León, P. (1987) Maize rayado fino and related viruses. Pages 213-233. in: Plant Viruses (Vol. 3). R. Koenig, ed. Plenum Publishing Corp.,New York.

Goodrick, B.J., Kuhn, C.W. & Hussey, R.S. (1991) Restricted systemic movement of

cowpea chlorotic mottle virus in soybean with nonnecrotic. Phytopathology 81: 1426-1431.

Landgale, J.A., Rothermel, B.A. & Nelson, T. (1988) Cellular pattern of photosynthetic gene expression in developing maize leaves. Genes & Development 2: 106-115.

Lazarowitz, S.G., Pinder, A.J.,, Damsteegt, V.D. & Rogers, S.G. (1989) Maize streak virus essential for systemic spread and symptom development. EMBO Journal 8: 1023-1032.

Leisner, S.M., Turgeon,R. & Howell, S.H. (1992) Long distance movement of cauliflower mosaic virus in infected turnip plants. Molec. Plant-Microbe Interac. 5: 41-47.

León,P. & Gámez, R. (1981) Some physicochemical properties of maize rayado fino virus. J.Gen.Virol. 56:67-75.

León, P.,Moreno,E.,Taylor, M.L., Silva, S., Ramírez, M. & Gámez, R. (1992) Capsid proteins of maize rayado fino virus (in preparation).

Lindbeck, A.G.C., Lewandowski, D.J., Culver, J.N., Thomson, W.W. & Dawson, W.O. (1992) Mutant coat protein of tobacco mosaic virus induces acute chlorosis in expanded and developing tobacco leaves. Molec. Plant-Microbe Interac. 5: 235-241.

Matthews, R.E.F. (1991) Disease Symptoms and Effects on Metabolism. Pages 381-385 in: Plant Virology. Third Edition. Academic Press. U.S.A.

Ramírez, P. & Hammond, R.W. (1987) Cloning of DNA copy of maize rayado fino virus (MRFV). Presented at hte International Congress of Plant Tissue Culture

Tropical Species. Sept. 21-25. Bogotá, Colombia.

Ramírez, P., Hammond, R.W., Karkashian, J. & Mora, M. (1988) Detection of maize rayado fino virus using biotynylated nucleic acid hybridization probes. Agrícola RIA 12

Rivera, C., Kozuka, Y. & Gámez, R. (1981) Maize rayado fino virus: Detection in salivary glands and evidence of increase in virus titre in the leafhopper vector Dalbulus maidis. Turrialba 31: 79-80.

Rodríguez-Cerezo, E., Gamble Klein, P. & Shaw, J.G. (1991) A determinant of disease symptom severity is located in the 3'-terminal noncoding region of the RNA of a plant virus. Proc. Natl. Acad. Sci. USA 88: 9863-9867.

Samuel, G. (1934) The movement of tobacco mosaic virus within the plant. Ann.Appl. Biol. 21:90-111.

Sharman, B.C. (1942) Development anatomy of the shoot of Zea mays L. Ann. Bot. 6: 245-284.

Turgeon, R. (1989) The sink-source transition in leaves. Ann. Rev.Plant Physiol. Plant Molec. Biol. 40: 119-138.

van Loon, L.C., van Kooten, O., Linders, E.G.A., Meurs, C., & Wijdeveld, M.M.C. (1990) Recognition and disease development in the tobacco-TMV system. Pages 311-328 in : Recognition and Response in Plant-Virus Interactions (Vol. 4). R.S.S. Fraser ed. Springer-Verlag. Germany.

van Telgen, H.J., van der Zaal, E.J., & van Loon, L.C. (1985) Evidence for an association between viral coat protein and host chromatin in mosaic-diseased tobacco leaves. Physiol. Plant Pathol. 26: 99-103.

van Sengbusch, P. (1985) Aminoseureaustausche und tertiaerstruktur eines proteins. Vergleich von mutanten des tabakmosaikvirus mit serologischen und physikochemischen. Z. Vererbungsl. 96: 364.

Verwoerd, T.C., Dekker, B.M.M. & Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acid Res. 17: 2362.

Ye, Z-H. & Varner, J. (1991) Tissue-specific expression of cell wall proteins in developing soybean tissue. Plant Cell 3: 23-37.

Table 1: Accumulation of MRFV-coat protein in proximal and distal part of MRFV-infected leaves

	PROXIMAL	DISTAL
Observations	101	96
Mean (a)	25,7	2,4 *
Variance	29.574,4	338,9
Standard Desv.	172	18,4
Standard Error	17,1	1,9

a: Data are expresed as ng coat protein/mg fresh tissue
*: Difference was significant with t-test at alfa= 0.05

Figure Legends

Fig.1 Symptoms induced by MRFV in the leaves of infected Z. mays L.Guararé at 15 days after insect inoculation. Only leaves 4-6 show mild and severe symptoms.

Fig.2 Accumulation of MRFV-coat protein in Z. mays L. Guararé in different leaves. Coat protein accumulation was measured by DAS-ELISA in healthy, exposed and infected plants. There were 2 replicates per mean value.

Fig.3 Accumlation of MRFV-coat protein in Z. mays L. Guararé in secondary roots. Coat protein accumulation was measured by DAS-ELISA in healthy, exposed and infected plants. There were 2 replicates per mean values.

Fig.4 Accumulation of MRFV RNA in healthy, exposed and infected leaves of Z. mays L. Guararé. Values were calculated by visual comparison of positive signals with dilutions of MRFV genomic RNA. Mean values for non-inoculated plants were substracted from those of virus-infected tissue.

Fig.5 Tissue print immunoblots showing the accumulation of MRFV-coat protein in tissues of healthy, exposed and infected Z. mays L. Guararé plants. Immunoblots show MRFV-coat protein in free-hand longitudinal and cross-section of (a) secondary roots, (b) stem and (c) leaves near to the stem .

Fig.6 Tissue print RNA blots showing accumulation of viral RNAs in tissues of healthy, exposed and infected Z. mays L. Guararé plants. RNA blots show MRFV RNA in free-handlongitudinal and cross-section of (a) secondary roots, (b) stem and (c) leaves near to

stem.

Fig. 7 Localization of MRFV viral RNA in cross-sections of infected maize leaves. Leaves expressing symptoms were fixed, embedded in paraffin, and cut into 10 μm sections. A ^{35}S-labeled anti-sense MRFV RNA probe (1.0×10^8 d.p.m./μg) was hybridized to plant sections for 16 hr at hybridization criteria of 42°C, 0.3 M Na^+, and 50% formamide. The slides were developed after 14 days exposure. The sections were stained with 1.0 % safranin-O. The photograph was taken using bright field microscopy. The hybridization signal can be seen over the vascular bundles in the leaf cross-section.

Fig. 1

Patzel?

Fig. 2

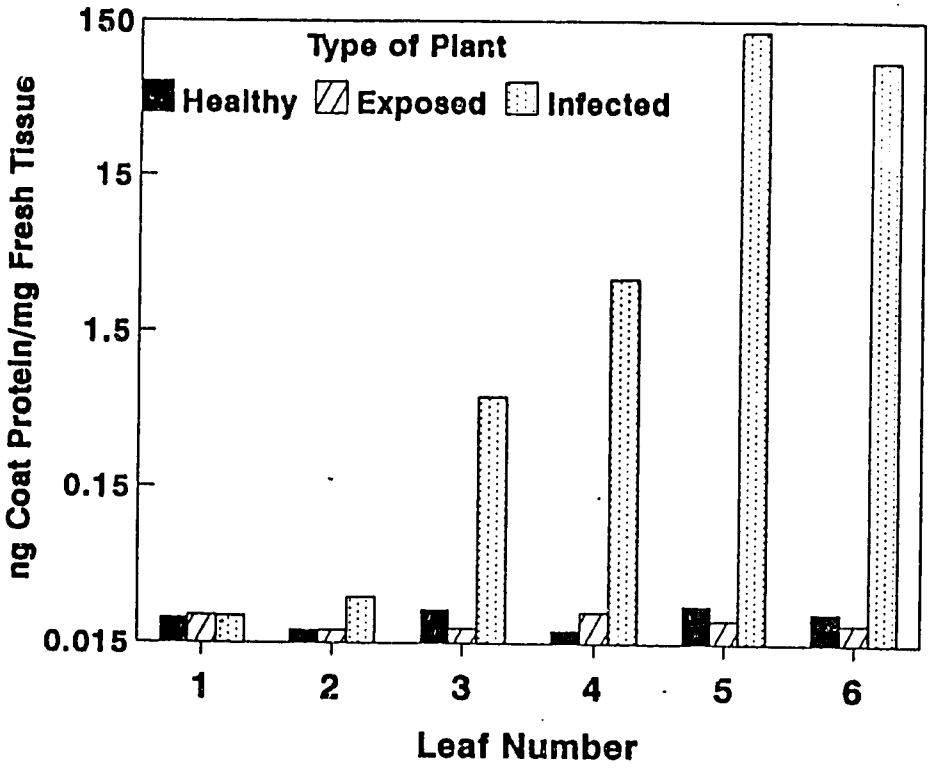
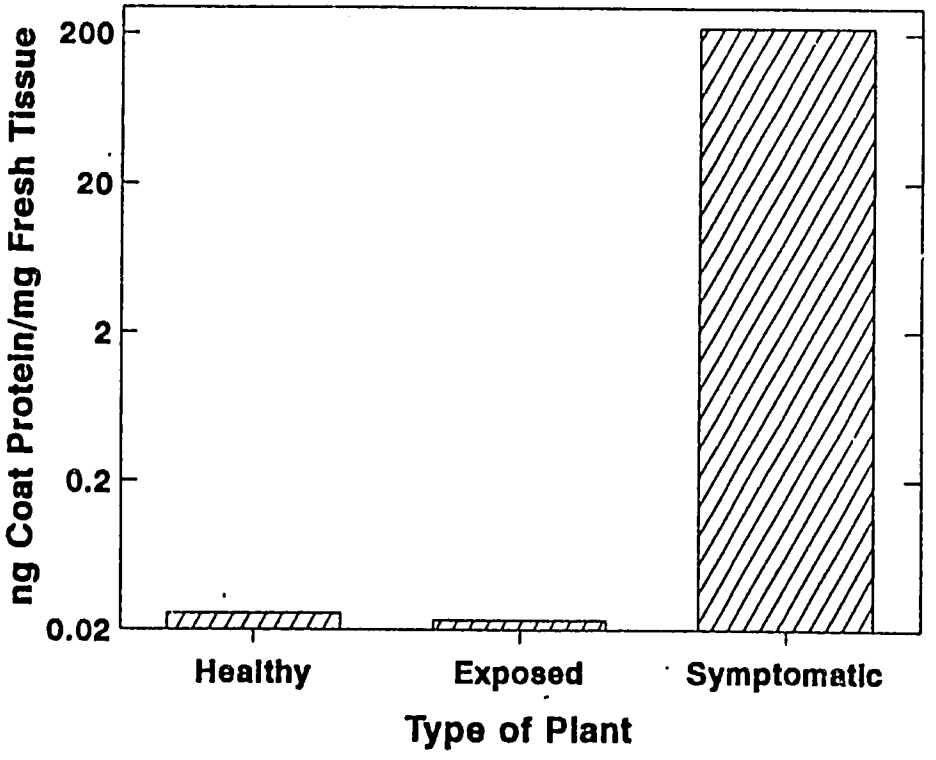
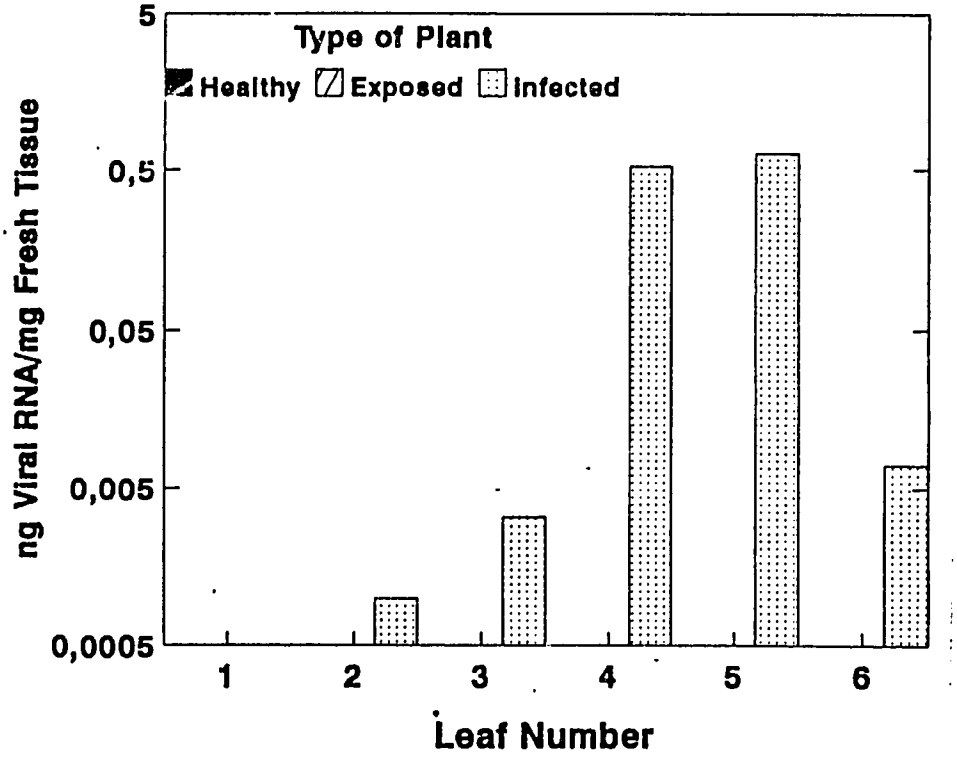

Type of Plant
Healthy
Exposed
Infected
150
15
1.5
0.15
0.015
ng Coat Protein/mg Fresh Tissue
1
2
3
4
5
6
Leaf Number

Fig. 3


ng Coat Protein/mg Fresh Tissue
200
20
2
0.2
0.02
Healthy
Exposed
Symptomatic
Type of Plant

7

Fig. 4



combine 2, 4

Fig. 5

Fig. 6

Incidence and Geographic Distribution of Maize Rayado Fino Virus (MRFV) in Latin America Identified by Dot-blot Hybridization

R. Kogel[1], R.W. Hammond[2], and P. Ramirez[1]

[1] Centro de Investaigacion en Biologia Celular y Molecular (CIBCM), Escuela de Biologia, Universidad de Costa Rica, San Jose, Costa Rica

[2] U,S. Department of Agriculture, Agricultural Research Service, Molecular Plant Pathology Laboratory, Beltsville, Maryland 20705 USA

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ABSTRACT

Kogel, R., Hammond, R. W., and Ramirez, P. 1995. Incidence and geographic distribution of maize rayado fino virus (MRFV) in Latin America identified by dot-blot hybridization. Plant Dis.

Samples of maize (*Zea mays* L.) exhibiting symptoms of the maize rayado fino virus (MRFV) were collected in eight Latin American countries during August - December of 1992, as part of a survey of virus distribution. Maize with MRFV symptoms were found in all the countries and life zones (12) sampled. All of the collected plants were tested for the presence of MRFV using hybridization with an MRFV cRNA probe. Different patterns of MRFV symptomatology were observed and the presence of the virus was confirmed by hybridization analysis.

Muestras de *Zea mays L.* con sintomas del virus Maize Rayado Fino Virus (MRFV) se colectaron en 8 paises de Latinoamerica durante el segundo de 1992, como un sondeo para determinar la distribucion y la abundancia de este virus. Maiz con sintomas del virus MRFV fue encontrado en todas los paises y las zonas de vida (12) en donde se busco. Todas las plantas colectadas fueron confirmadas con el methodo de hibridacion para la presencia de MRFV. Diferentes patrones de sontomas fueron encontrados y confirmados con el analisis de hibridacion.

The leafhopper-borne maize rayado fino virus (MRFV) is the only known indigenous virus of maize in Meso America (9). MRFV is the best characterized member of the marafivirus group of plant viruses, which includes oat blue dwarf virus and Bermuda grass etched line virus (5). MRFV, which appears to be restricted to the Americas, is widespread and increasingly important in tropical areas (6,8). It possesses small isometric particles, ca. 30 nm in diameter, and a single-stranded RNA genome with a molecular weight of 2.0 - 2.1 x 10^6 daltons (16). The virus is able is able to replicate in both maize and its insect vector (*Dalbulus maidis* Delong and Wolcott) (19). MRFV often occurs in field infections associated with mollicutes (corn stunt spiroplasma and maize bushy stunt phytoplasma [MLO]) which are transmitted by the same vector (10). There is a close association between *D. maidis* and MRFV and their maize host (11). Maize is considered the only natural host in areas where MRFV and its leafhopper vector are endemic (7). It has been proposed that MRFV, *D. maidis*, and *Zea mays* L. coevolved in a triad in which the parasitic members (virus and insect) display highly specialized interactions (9).

MRFV and *D. maidis* have successfully exploited the capacity of *Z. mays* to adapt to a broad range of environments. Both the virus and insect have been found in maize fields from sea level to altitudes of over 3500 meters and from the southern United States through Mexico and Central America to Northern Argentina and Uruguay in South America (7,8).

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Only preliminary information is available about the existence of strains of MRFV. The Brazilian corn streak virus (13), the maize rayado colombiano virus (17) and MRFV are all strains of the same virus (5, 6, 8).

MRFV was first recognized in El Salvador and Costa Rica over fifteen years ago and has since been detected in all Central American countries, including Guatemala, Honduras, Nicaragua, and Panama (3, 4, 8), Mexico (8, 20), the southern USA (1), Colombia (17), Peru (8), Venezuela (15), Brazil (13), and Uruguay (8).

This work describes the most comprehensive survey to date of the incidence and distribution of MRFV in maize fields of 8 Latin-American countries during August - December 1992 using molecular techniques. The distribution and different symptomatology patterns of MRFV in field infections are reported.

MATERIALS AND METHODS

Sample collection. Maize leaf samples were collected in eight Latin-American countries (Mexico, Guatemala, Honduras, Costa Rica, Colombia, Venezuela, Peru, and Bolivia) during August, September, October, November, and December 1992 (Table 1). In each country, an extensive collection was made according to the possibility of safe travel within that country and the accessibility of maize plantations. Within each country, various geographic zones were defined by physical distance and geographic barriers in order to group the collecting sites. Ecological zones were defined using Holdridge's parameters (12). For each collecting site, a passport

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card describing made some characteristics of the environment, geography of the crop, and the plant symptoms was made. A photographic record of each plant sampled and a close-up of the symptoms was maintained.

Material was collected from the basal part of the leaves which most frequently expressed symptoms. The leaves sampled were preserved by placing them in paper bags with paper sheets separating the maize leaves. In the first three to seven days after sampling, the leaves were air dried. The dried tissue was then packed in plastic bags and stored in a suitcase for a period of 2 to 3 months. The samples were lyophilized upon arrival to the laboratory in Costa Rica, followed by storage at -70 C.

Samples of corn plants infected with the Guapiles (Costa Rica) isolate under controlled conditions and uninfected corn plants, grown in the greenhouse, were used as positive and negative controls, respectively, for the laboratory experiments.

Nucleic acid extraction. Lyophilized corn leaves were ground to a powder with a mortar and pestle in liquid nitrogen, and nucleic acids were extracted by a modification of the direct phenol method (2). Briefly, the leaf powder was homogenized with 10 ml of 0.1 M Tris,, pH 8.9 containing 0.1 M sodium diethyl-dithiocarbamic acid, 0.1 M sodium chloride, 0.01 M EDTA, 0.005 M dithiothreitol, and 1% SDS, 10 ml buffer-saturated phenol (Life Technologies,Gibco-BRL, Gaithersburg, MD) and an equal volume of chloroform. The mixture was shaken for 10 minutes and the aqueous and organic phases were separated by centifugation for 10 min at 5000 x g.

Total nucleic acids were precipitated from the aqueous phase with 0.33 vol of 7.5 M ammonium acetate, pH 5.2 and 3 vol of ethanol. Polysaccharides were removed by adding equal volumes of 2.5 M K_2HPO_4, pH 8.0, and methoxyethanol to the water-suspended nucleic acids.

After the addition of 0.25 vol of 1% cetyltrimethylammonium bromide (CTAB) to the separated upper phase from the previous step, 2 hr of storage on ice followed. Nucleic acids were pelleted by centrifugation for 10 min at 5000 x g. The pellet was washed three times with 70% ethanol/0.1 M sodium acetate pH 5.2 and was resuspended in 200 ul of sterile, double-distilled water and re-precipitated with 2.5 vol of ethanol and 0.1 vol of 3 M sodium acetate, pH 5.2. The final pellet was resuspended in 200 ul sterile water.

Hybridization analysis. Hybridization of 86 samples was performed using an RNA probe of the capsid protein gene, produced by *in vitro* transcription from the 4,65 clone (18). The probe was labelled with ^{32}P-UTP (Amersham Corp., Arlington Heights, IL; specific activity = 800 Ci/mmol).

Nylon membranes (Nytran, Schleicher and Schuell, Keene, NH) which had been prewet with water, 2 X SSC (1 X SSC = 3.0 M NaCl, 0.03 M sodium citrate, pH 7.0) and air-dried, were spotted with approximately 6 - 7 ug of total nucleic acids acids. Nucleic acids were crosslinked to the membrane in an Ultra Violet light Stratalinker (Stratagene Cloning Systems, La Jolla, CA).

Prehybridization was performed with a buffer consisting of 6

X SSC, 4 X Denhardts (1 X = 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.1 % SDS, and 100 ug/ml denatured calf thymus DNA at 65 C for 20 min.

For the hybridization, 1 ul (approx. 1,000,000 cpm) of the 4,65 RNA probe was added per ml of prehybridization mix, followed by incubation overnight at 65 C. The membranes were washed at room temperature with 2 X SSC and 0.2% SDS for 30 min, followed by treatment with RNase A (final concentration of 1 ug/ml in 2 X SSC) for 15 min at room temperature, followed by a final wash with 0.1% SSC and 0.1% SDS for 30 min at 50 C. Autoradiography was performed by exposing the filter to Kodak X-AR film.

RESULTS

Preservation of the samples. From the 225 samples collected, less than 10 were lost to rot on the way back to the laboratory in Costa Rica (all of them from an area in Honduras).

Geographical distribution. MRFV was detected in the 8 countries where the sampling was done. Of the 34 geographic zones that were sampled, 28 (82%) had maize plants exhibiting symptoms of MRFV infection and 6 (18%) did not (Table 1). Of the 94 collecting sites sampled, MRFV symptoms were detected in 68 (72%) of the sites, while in 26 (28%) symptoms of MRFV were not found (Table 1).

Maize plants exhibiting symptoms of MRFV infection were found in a very wide distribution, from latitude 20 north to latitude 18 south, and from longitude 100 west to longitude 63 west, and from sea level to 3100 meters above sea level (Table 2). MRFV was found

in very different zones: coast zone (tropical, basal wet forest in Patalan, Mexico); mountain zone (tropical, high montane forest in Urubamba, Peru); desertic zone (subtropical, basal desert in Chincha, Peru); humid tropics zone (tropical, basal rain forest in Guapiles, Costa Rica); high land savannah (tropical, lower montane dry forest in Bogota, Colombia) and Amazonic zone (tropical, wet forest in Santa Cruz, Bolivia).

Symptomatology. Symptoms of MRFV infection are characterized as distinct, conspicuous, small chlorotic spots that develop at the base and along the veins of young leaves in characteristic stippled stripes (7)(Fig. 2). Many variations of the expected symptomatology were found, including what is considered the symptoms of maize rayado colombiano virus (Fig. 2C) which was found in ICA-Mosquera, Bogota, Condinamarca (identified by Martinez-Lopez, 17), and in ICA-palmira, Valle del Cauca (identified by Franci B\Varon de Agudelo). In both places, MRFV symptoms were found in the same maize germplasm next to rayado colombiano virus.

An atypical expression pattern was observed in plants found in Nueva Concepcion, Guatemala and Cochabamba, Bolivia, where in plants with very severe infection, the symptoms are stronger in the older leaves and in the younger leaves the symptoms were concentrated at the apex instead of the base until the symptoms disappear in the youngest leaves. All of these samples were positive in the hybridization analysis.

In Mexico and Central and South America, virus incidence may range from 0 - 40% in most locations, but up to 100% in others.

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Yield losses also frequently vary around 40 - 50% of the weight of the mature ear in local genotypes, but reach nearly 100% in recently introduced improved cultivars (7). The susceptibility among maize cultivars to MRFV is very variable (4, 5, 6, 7, 17). Disease symptoms in the more sensitive genotypes are characterized by numerous short and long, chlorotic stripes, wilting of young plants, and general chlorosis and stunting. In the more tolerant genotypes, the characteristic striping gradually becomes milder and may even disappear in the new, youngest, leaves (4, 5, 17). The expression of the symptoms of MRFV in the plant is concentrated at the base of the leaves, generally with an increase of severity toward the younger leaves.

Hybridization analysis. Of the 255 samples collected, 103 were chosen as representative samples from the different locations for hybridization with the MRFV probe. Seventy-five (73%) were positive and 28 (27%) were negative (Table 2). Figure 1 shows a representative dot blot and shows how the results were categorized (+++, ++, +, and -).

DISCUSSION

The method of air drying and preserving the maize leaves in paper bags for further nucleic acid extraction was adequate for the most part, economical, and practical for long collecting field trips. The fact that we had negative hybridization results for samples that visually appeared to be infected with MRFV may be explained by either some degradation of the samples during

preservation or that symptoms were produced by another pathogen.

Even though MRFV seems to be very well distributed in Bolivia, this is the first report of the incidence of the virus in this country by any means other than symptomatology.

MRFV is widely distributed throughout Latin America and it appears to be present in all regions where there is maize in abundance and its insect vector D. maidis occurs, regardless of the latitude or ecological zone. The variety of ecosystems under which the virus and vector are found include nearly 20 different types of life zones, as defined by Holdridge (12), ranging from deserts through savannahs, deciduous and montane forests to lowland rain forests (9). MRFV occurs in all maize growing regions of Costa Rica, which includes eleven different vegetation zones in basal, premontane, lower-montane and montane altitudinal belts. The 4 zones differ in altitude, annual mean temperature, amount and seasonal distrubution of rainfall, light intensity, soil conditions and physiography (12)..The presence of MRFV in a specific habitat is probably more dependent on the ability of D. maidis to survive in those conditions, and its ability to migrate short (14) or long (21) distances, rather than the effect of those same conditions on the viability of the virus. Wind assisted migrations may disperse insects from areas where maize is grown throughout the year to areas where the crop is grown only seasonally, eg. for distances of several hundred kilometers (21), to areas such as the high Peruvian Andes, Mexican Mesa Central and southern United States (9). In these areas, as in the southern United states, low temperatures and

other ecological conditions are unfavorable for the survival of the insect between planting seasons (9). This is consistent with the records and observations made by local farmers that the disease appears only in certain years, or that the disease may be mild and at other times very damaging.

Another possible explanation for the wide distribution of MRFV is that corn is a very abundant crop and, although a wide variety of germplasm is grown, the microhabitat developed within the crop field is similar under many climatic conditions (9). Thus, MRFV and D. maidis would not require any exceptional ability to adapt to the different ecological conditions of the diverse zones where they exist (9).

No maize germplasm is yet known to be resistant to MRFV infection (22). Even though we report here some negative hybridization results for some of the germplasm, all of these samples appeared to display symptoms of MRFV infection. As these were field samples, no duplicate data exists to confirm these findings.

Acknowledgments.

This research was supported in part under grant number 936-5542.13 of the project number 9.198, Program in Science and Technology Cooperation, Office of the Science Advisor, U. S. Agency for International Development.

We would like to acknowledge the collaboration we received from the following individuals during the collection of the maize

samples in the different Latin American countries: Dr. Dan Jeffers, CIMMYT, Mexico; Dr. Francisco Cárdenas, Chapingo, Mexico; Dr. Mario Roberto Fuentes López, Ing. Humberto Alareon Lucero and Ing. Jose Luis Quemé de León, Guatemala; Dr. Arnoldo Bejarano, CENIAP, Venezuela; Luis Pineda y Jeannette, Medillín, Colombia; Francia Varon de Agudelo, Valle del Cauca, Colombia; Guillermo Urelo y Pedro Tujo Infante, Cajamarca, Peru; Ing. A. Villantoy Palomino, Ayacucho, Peru; Dr. Gonzalo Avila y Marcia Céspedes, Cochabamba, Bolivia. Very special thanks to Dr. Upali Jayasinghe, CIP, Peru; María Belén Delgado, Venezuela; Dr. Carlos Días, Medellín, Colombia, and Monica Olvera for their attentions, information and hospitality, and Federico Albertazzi for advice on identification and collection of MRFV, at CIBCM - UCR, Costa Rica.

We also with to thank Shanta Rishi, Rita Mathur, and April Clinkscales for technical assistance at USDA/ARS, Beltsville, MD, USA and J. Hammond, H.-T. Hsu, and E. Podleckis for critical review of the manuscript.

LITERATURE CITED

1. Bradfute, E., Nault, R., Gordon, T., Robertson, C., Toler, W., and Boothroyd, W. 1980. Identification of maize rayado fino virus in the United States. Plant Dis. 64:50-53.

2. Diener, T. O. (ed.) 1979. Viroids and viroid diseases. Wiley-Interscience, New York.

3. Gámez, R. 1969. A new leafhopper-borne virus of corn in Central America. Plant Dis. Reporter. 53:929-932.

4. Gámez, R. 1973. Transmission of rayado fino virus of maize (Zea mays L.) by Dalbulus maidis Delong & Wolcott. Ann. Appl. Biol. 73:285-292.

5. Gámez, R. 1980. Maize rayado fino virus. CMI/AAB Descriptions of Plant Viruses. 220, 4pp.

6. Gámez, R. 1980. Rayado fino virus of maize in the American tropics. Tropical Pest Management. 26:26-33.

7. Gámez, R. 1983. The ecology of maize rayado fino virus in the American Tropics. In: Plant Virus Epidemiology (Plumb, R. T., and Thresh, J. M., eds.) Blackwell Scientific Publications, Oxford. pp. 267-275.

8. Gámez, R., Kitajima, E. W., and Lin, M. T. 1979. The geographical distribution of maize rayado fino virus. Plat Dis. Reporter. 63:830-833.

9. Gámez, R., and León, P. 1983. Maize Rayado Fino Virus: Evolution with plant host and insect vector. In: Current Topics in Vector Research, Vol. I. (Harris, K. F., ed.) Praeger Publishers, New York. pp. 149-168.

10. Gámez, R., and León, P. 1985. Ecology and evolution of neotropical leafhopper-virus-maize association. In: The leafhopper and planthopper. (Nault, L. R., and Rodriguez, J. G., eds.) J. Wiley and Sons, Inc. New York. pp. 331-350.

11. Gámez, R., and Saavedra, F. 1986. Maize rayado fino: A model of a leafhopper-borne virus disease in the Neotropics. In: Plant Virus Epidemics: Monitoring, Modelling and Predicting Outbreaks. (McClean, G. D., Garrett, R. G., and Ruesink, W. G., eds.) Academic Press, Australia pp. 315-326.

12. Holdridge, R. 1978. Ecología basada en zonas de vida. Instituto interamericano de Ciencias Agrícolas, San José, Costa Rica

13. Kitajima, W., Yano, T., and Costa, S. 1976. Purification and intracellular location of isometric particles associated with the Brazilian corn streak virus infection. Ciencia e Cultura 28: 427-

430.

14. Larsen, K., Nault, L., and Moya-Raygoza, G. 1992. Overwintering biology of Dalbulus leafhoppers (HomopteraL Cicadellidae): Adult populations and drought hardiness. Environ. Entomol. 21: 566-577.

15. Lastra, R., and de Uscategui, C. 1980. El virus rayado fino del maiz en Venezuela. Turrialba. 30: 405-408.

16. León, P. and Gámez, R. 1981. Some physiochemical properties of Maize rayado fino virus. Journal of General Virology. 56: 67-75.

17. Martínez-López, G. 1977. New maize virus diseases in Colombia. Proceedings of the International maize Virus Disease Colloqium and Workshop (Williams, L., Gordon, D., and Nault, R., eds.) Ohio Agricultural Research and Development Center, Wooster. pp. 20-29.

18. Ramirez, P., Hammond, R. W., Karkashian, J., and Mora, M. 1990. Production of biotinylated nucleic acid probes for detection of maize rayado fino virus. Revista de Invest. Agr. 22: 50-55.

19. Rivera, C., and Gámez, R. 1986. Multiplication of maize rayado fino virus in its leafhopper vector Dalbulus maidis. Intervirology. 25: 76-82.

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20. Rocha-Peña, M. 1981. Algunos aspectos relacionados con el virus del rayado fino del maíz en Mexico. MSc. Thesis. Colegio de Posgraduados, Chapingo, Mexico.

21. Taylor, R. A. J., Nault, L. R., and Styer, W. E. 1993. Experimental analysis of flight activity of three Dalbulus leafhoppers (Homoptera: Auchenorrhyncha) in relation to migration. Annals of the Entomological Society of America. 86: 655-667.

22. Toler, R. 1985. Reactions of maize (Zea mays) accessions to maize rayado fino virus. Plant Disease. 69: 56-57.

Table 1. Number of geographic zones and collecting sites in which symptoms of MRFV infection were observed.

COUNTRIES	GEOGRAPHIC ZONES		COLLECTING SITES	
	MRFV Symptoms present	absent	MRFV Symptoms present	absent
Mexico	8	1	16	5
Honduras	2	0	4	2
Guatemala	3	1	5	5
Costa Rica	3	1	7	5
Colombia	4	0	6	0
Venezuela	1	1	3	2
Peru	5	1	21	3
Bolivia	2	1	6	4
Number of sites	28	6	68	26
Percentage	82%	18%	72%	28%
TOTAL	34 geographic zones		94 collecting sites	

1 Table 2. List of samples collected in eight Latin American
2 countries in August - December, 1992.

3 Footnotes:
4 [1]Geographic location of the collecting site is defined by latitude,
5 longitude and altitude (m.a.s.l. = meters above sea level).

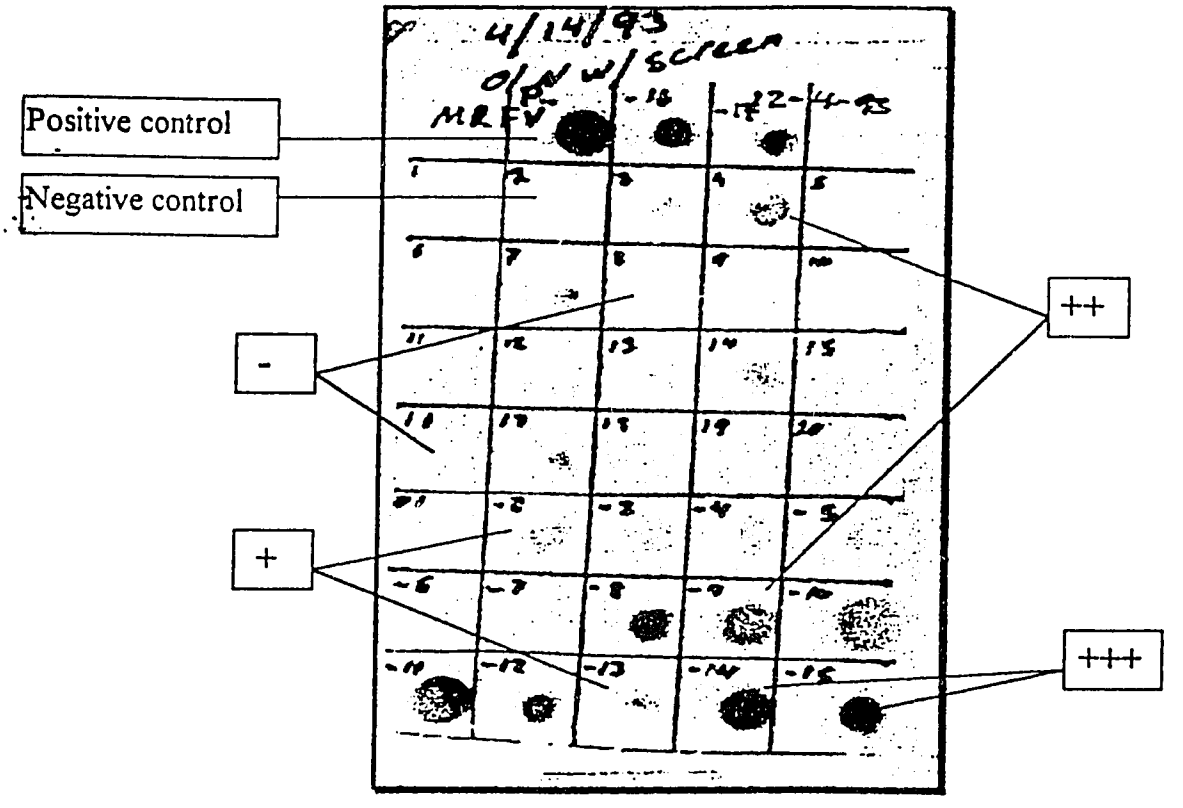
6 [2]Results of hybridization analysis using the cRNA probe are
7 expressed as defined in Figure 1.

#	Country	Collecting site	ALTITUDE	LATITUDE	LONGITUDE	HYBRIDIZATION
			m.a.s.l			ADN 4,55 probe
01	Mexico	Francisco Villa, Veracruz	50	20° 18'	97° 18'	+++
02		Ojo de Agua, Veracruz	100	20° 18'	97° 18'	++
03		Cotaxtla, Veracruz	16	18° 48'	96° 21'	-
04		Ciudad Mendoza, Orizaba, Ver.	1400	18° 45'	97° 12'	++
05		Ciudad Mendoza, Orizaba, Ver.	1400	18° 45'	97° 12'	+++
06		Zumbilla, Puebla	2100	18° 39'	97° 18'	+
07		Acatepec, Puebla	2000	18° 15'	97° 33'	+++
08		Agua Dulce, Oaxaca	1700	17° 54'	97° 48'	-
09		Chila, Puebla	1700	17° 57'	97° 54'	+++
10		Chila, Puebla	1700	17° 57'	97° 54'	+
11		Chila, Puebla	1700	17° 57'	97° 54'	+
12		Teloicapan, Guerrero	1500	18° 22'	99° 54'	+
13		Coyuca, Guerrero	400	18° 18'	100° 48'	+
14	Honduras	Sambrano, Francisco Morazan	1550	14° 17'	87° 25'	+++
15		Ojo de Agua, El Paraiso	620	14° 04'	86° 53'	-
16		Ojo de Agua, El Paraiso	620	14° 04'	86° 53'	++
17		Ojo de Agua, El Paraiso	620	14° 04'	86° 53'	+
18		Chichicaste, El Paraiso	500	14° 04'	86° 23'	+
19		Santa Maria, El Paraiso	460	14° 09'	86° 17'	+
20	Guatemala	La Máquina, Suchitepéquez	75	14° 16'	91° 35'	-
21		La Máquina, Suchitepéquez	75	14° 16'	91° 35'	+
22		La Máquina, Suchitepéquez	75	14° 16'	91° 35'	-
23		Nueva Concepción, Escuintla	100	14° 10'	91° 10'	-
24		Nueva Concepción, Escuintla	100	14° 10'	91° 10'	+
25		Santa Lucia, Sacatepéquez	2000	14° 34'	91° 40'	+
26		Mita, Jutiapa	800	14° 21'	89° 43'	+++
27	Colombia	Uyamal, Antioquia	500	6° 30'	75° 48'	+
28		Uyamal, Antioquia	500	6° 30'	75° 48'	-
29		Santa Fe, Antioquia	500	6° 33'	75° 48'	+
30		Santa Fe, Antioquia	500	6° 33'	75° 48'	+
31		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+
32		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	-
33		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	++
34		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+
35		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	-
36		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	-
37		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	++
38		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	-
39		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+++
40		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+++
41		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	++
42		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+
43		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	-
44		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	+++
45		Ica-Bello, Medellín, Antioquia	1440	6° 19'	75° 21'	++
46		Rio Negro	2120	6° 10'	75° 23'	+
47		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	+
48		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
49		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
50		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	++
51		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	+
52		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
53		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	++
54		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
55		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	++
56		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
57		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
58		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
59		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
60		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	+++
61		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
62		ICA- Palmira, Valle del Cauco	1000	3° 32'	76° 41'	-
63		ICA-Mosquera, Condinamarca	2400	4° 41'	74° 12'	+++

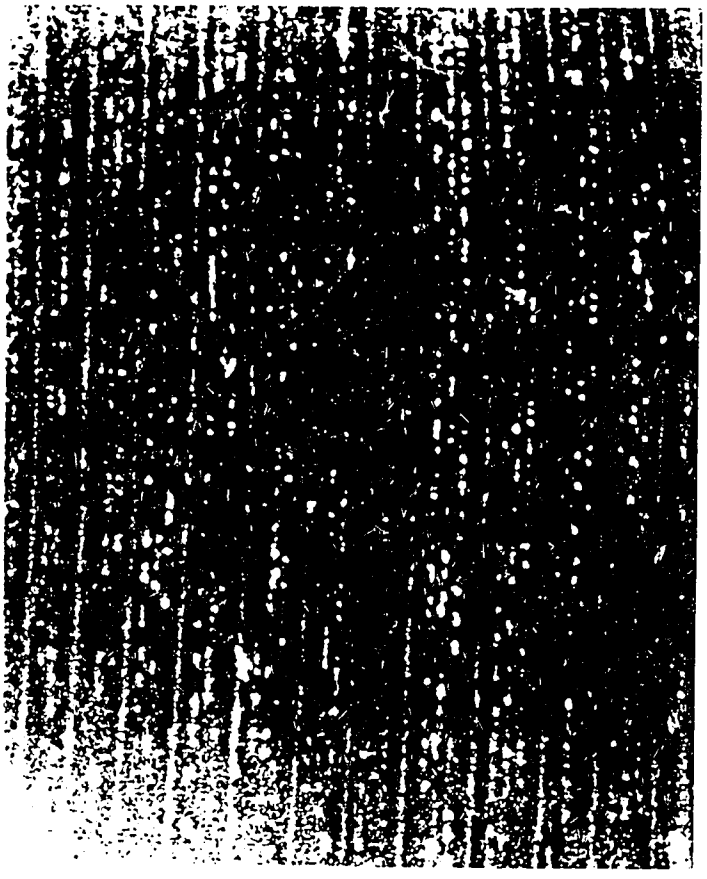
64		ICA-Mosquera, Condinamarca	2400	4° 41'	74° 12'	+++
65		ICA-Mosquera, Condinamarca	2400	4° 41'	74° 12'	-
66	Venezuela	S. Juan de los Morros, Guarico	300	9° 45'	67° 21'	+
67		S. Juan de los Morros, Guarico	300	9° 45'	67° 21'	+
68		CENIAP-Maracay, Aragua	455	10° 17'	67° 36'	+++
69		CENIAP-Maracay, Aragua	455	10° 17'	67° 36'	-
70		CENIAP-Maracay, Aragua	455	10° 17'	67° 36'	+++
71		CENIAP-Maracay, Aragua	455	10° 17'	67° 36'	+
72	Perú	Chincha-INIAA, Ica	280	-13° 30'	76° 08'	+++
73		Guanami, Ica	600	-13° 55'	75° 38'	+
74		Nazca, Ica	250	-14° 50'	74° 57'	+
75		Jesús, Cajamarca	2500	-7° 12'	78° 30	-
76		Jesús, Cajamarca	2500	-7° 12'	78° 30	+++
77		Jesús, Cajamarca	2500	-7° 12'	78° 30	+++
78		Jesús, Cajamarca	2500	-7° 12'	78° 30	+
79		Namora, Cajamarca	2850	-7° 15'	78° 20'	+++
80		Namora, Cajamarca	2850	-7° 15'	78° 20'	+++
81		Huayubamba, Cajamarca	2000	-7° 17'	78° 17'	++
82		Huayubamba, Cajamarca	2000	-7° 17'	78° 17'	+++
83		San Marcos, Cajamarca	2000	-7° 20'	78° 15'	++
84		San Marcos, Cajamarca	2000	-7° 20'	78° 15'	++
85		Urubamba, Cuzco	3100	-13° 23'	72° 08'	++
86		Ollantaytambo, Cuzco	2760	-13° 12'	72° 17'	++
87		Yucay, Cuzco	2900	-13° 20'	72° 05'	-
88		Muyurina, Ayacucho	2420	13° 00'	74° 10'	+++
89		Valle de Huatatas, Ayacucho	2600	13° 00'	74° 10'	+++
90		Valle de Chaceo, Ayacucho	2600	13° 00'	74° 10'	+++
91		Camcán, Ayacucho	2720	13° 00'	74° 10'	+++
92	Bolivia	Univ. Agraria, Cochabamba	2560	-17° 25'	66° 05'	+++
93		Pairumani, Cochabamba	2560	-17° 25'	66° 15'	+++
94		Pairumani, Cochabamba	2560	-17° 25'	66° 15'	++
95		Vallecito, UAA, Santa Cruz	2560	-17° 25'	66° 15'	+
96		Torno, Santa Cruz	450	-18° 02'	63° 25'	+++
97	Costa Rica	Invernadero, Guápiles	1200	10° 15'	83° 50'	++
98		San Rafael de Alajuela	800	9° 52'	84° 15'	-
99		San Rafael de Alajuela	800	9° 52'	84° 15'	+++
100		San Rafael de Alajuela	800	9° 52'	84° 15'	-
101		Fabio Baurit, Alajuela	800	10° 00'	84° 17'	+++
102		Zarcero	1500	10° 12'	84° 25'	+
103		Zarcero	1500	10° 12'	84° 25'	++

Figure 1. Dot blot analysis of equivalent amounts of nucleic acids extracted from maize samples using the ^{32}P-labelled 4,65 cRNA probe, as described in Materials and Methods. The categorization of signal intensity on the autoradiogram is shown on the figure as : +++, when the hybridization signal was similar to a highly infected positive control; ++, when a relatively strong hybridization signal was observed; +, when a weak signal was observed; -, when the signal was similar to the negative control.

Figure 2. Typical and atypical symptoms observed in field infections of maize that testing positive for MRFV. A. Typical moderate to severe symptoms of MRFV infection. B. Atypical symptoms of MRFV infection in maize leaves collected from Ciudad Mendoza, Veracruz, Mexico. C. Symptoms of MRFV infection in maize from Palmira, Colombia (identified as Rayado Colombiana virus by F. Varón de Agudelo and G. Martinez-Lopez).


Positive control
Negative control
-
+
++
+++

A



B

C

Evaluation of maize germplasm for resistance to maize rayado fino virus

Pedro I. Bustamante, Rosemarie Hammond and Pilar Ramirez.

First author: John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK; second author: USDA-ARS, Molecular Plant Pathology Laboratory, Beltsville, Maryland 20705-2350, USA and third author: Centro de Investigacion en Biologia Celular y Molecular (CIBCM), Universidad de Costa Rica, San Jose, Costa Rica.

Acceptance date:

ABSTRACT

Maize rayado fino virus (MRFV) causes severe yield losses in maize (*Zea mays L.)* in the Latin American countries. Yield losses of 40-50% are recorded on indigenous genotypes, but may reach 100% in some newly developed cultivars. Tweny different maize genotypes were obtained from Centre for the Improvement of Maize and Wheat (CIMMYT Mexico), Estacion Experimental Fabio Baudri(EEFB-Alajuela) and Consejo Nacional de la Produccion (CNP) from Costa Rica. Enzyme-liked immunosorbent assay (ELISA) and a SLOT-BLOT) hybridisation assays were developed together with a symptomatology scale to evaluate the responses of the maize genotypes to MRFV infection under screenhouse conditions. Two genotyes of the CIMMYT germ plasm collection (2980-93 and 3974) seem to be resistant to MRFV infection under the conditions tested. These genotypes showed low symptomatology and CP MRFV coat pattern concentration, but the levels of viral RNA did not correlate with the severity of the symptoms associated with the infection.

Maize rayado fino virus (MRFV) is the type member of the Marafivirus group of plant viruses which includes oat blue dwarf virus (OBDV) and Bermuda grass etched line virus (BGELV) and possess isometric particles, ca 30 nm in diameter containing a single stranded RNA of 2 X 10^6 mol. wt. and two distinct capsid proteins of 2.2 and 2.8 X 10^6 mol. wt. (8,10,11,13,18). MRFV is transmitted by the leafhopper, *Dalbulus maidis* (De Long & Wolcott) in a circulative-ppropagative maner (9,11,28). The virus is acquired from infected plants and inoculated to healthy plants after feeding period of 6-8 hr (9). Before transmission occurs MRFV undergoes a long incubation period of 8-37 days, with a mean of12-17 days at 25°C (13). The virus is not mechanically transmissible and in the field *D. maidis* is the main vector although other leafhoppers, *D. elimatus* (Ball), *Baldulus tripsaci* (Kramer & Whitcomb), *Sdrellus bicolor* (Van Duzee), and *Graminella nigrofons* (Forbes), experimentally transmit the virus (24).

MRFV is the only autochthonous virus of maize presently known to exist solely in this crop in its native center of origin

(10,12,29). It has been reported that MRFV may induce losses between 10-40% of the weight of the mature car in local genotypes, but can reach nearly 100% in recently introduced cultivars (12,13). The susceptibility among maize cultivars to MRFV is very variable. The symptomatology in the susceptible genotypes are characterized by numerous short and long chlorotic stripes, wilting of young plants, general chlorosis and stunting. Lethal effects have been reported in Colombia and El Salvador (13). The expression of the symptoms of MRFV in the plant is concentrated at the base of the leaves, with a increase of severity toward the younger leaves (10,13,21).

There are no satisfactory control measures for MRFV (10) and no inmmune genotypes have been detected among representative races of maize. Furthermore, no commerically acceptable level of resistance was found in North American germplasm (33). Locally adapted Central American materials appeal in general to be more tolerant to the virus and to show lower degrees of incidence within a population (12). For that reason, identifying new sources of resistance and finding suitable methods for characterizing MRFV resistance is very important.

The symptomatology assay, which involves visual inspection of symptom development, is currently the most common method of screening germplasm for resistance to plant viruses. However, observation of symptom expression in an assay for viral diseases should not be used exclusively because, as witn as many viral diseases, the degree of symptom severity is not always proportional to viral replication within the host (35). Two virus diagnostic techniques, enzyme-linked immunosorbent assay (ELISA) and slot-blot hybridization, in conjunction with a symptomatology assay, have been used to evaluate wild wheat species for resistance to wheat streak mosaic virus (WSMV) (32).

Using assays that determine the viral nucleic acid content in the host (SLOT-BLOT hybridization) and viral capsid protein content (ELISA), in addition to symptomatology assays, will provide a more accurate method of screening maize genotypes and germ plasm for MRFV resistance.

The first objective was to establish a methodology to evaluate the response of 20 different genotypes of maize to MRFV infection. The second objective was to determine if symptomatology, viral protein production as detected by ELISA, and viral RNA replication as detected by slot-hybridization were correlated and what assay or combination of assays best determined resistant germplasm.

Material and methods

A selection of maize landraces was obtained from International Centre for the Improvement of Maize and Wheat (CIMMYT Mexico). In addition, two hybrids were obtained for from Fabio Baudrit Experimental Station (EEFB-Alajuela), University of Costa Rica and three maize cultivars from Naclonal Production Council (CNP), San Jose, Costa Rica. See Table 1 for details of the different genotypes tested. All seeds from the different sources were assumed to be homogenous. Every accesion is periodically grown out

and selfed as a part of the CIMMYT germ plasm maintenance program. Genotypes representatives of the different races of maize were selected according the information provided by Bretting et al., (3).

Planting method in the first evaluation. The experimental design was a randomized complete block with 20 different genotypes and five replications grown under screenhouse conditions. Two seeds were sown per hill and later thinned to one plant before virus infestation. Fertilizers applied ha^{-1} were 90kg of N. 60kg of P_2O_5, and 30 kg of K_2O. The soil was a tropical alfisol (rain forest), 10 individual plants were used for each replication by exposing to infesting five with viruliferous *D. maidis* and kept under screenhouse conditions at CIBCM, and five with non-exposed *D. maidis* and kept in similar conditions at the Agronomy Faculty of University of Costa Rica. All plants were artifically infested, with 10 to 12 either viruliferous or non exposed leafhoppers placed in the whorl of individual plants at third to fourth days after emergence. The leafhoppers were kept for about 3 days, called transmission period (TP), after that the vector was removed and the plants fumigated with Tamaron to prevent the reinoculation of the virus and the plants were kept under 14 hr light and 22 $\pm 1°C$. Fluorescent lightling (10,800 lux) was used to complement natural light. Plants were watered regularly and examined for symptoms two days after inoculation and every other day thereafter for 15 days. A total of 1,000 plants were analized in this first evaluation.

Planting method in the second evaluation. In the second phase the genotypes assayed were Guarare. HC-78 (considered "susceptible") and 3974 and 2980-93 (considered "resistant") according the symptomatology showed during the first evaluation. Each plant was labelled as well as the group of insects with which each one was inoculated. For details, see Fig. 2.

During this second phase, it was a particular interest to determine if all those groups of insects exposed to the virus would infect and induce symptoms in genotypes Guarare and HC-78; in the first as well as in the thired transmission periods but did no do for genotypes 3974 and 2980-93 in the second transmission period. A total of 180 plants were analyzed in the second evaluation.

Inoculum preparation. An isolated of MRFV was obtained from the Guapiles location of Costa Rica (Guapiles-MRFV isolate) and was maintained on a susceptible corn hybrid, with frequent transfer. The inoculum procedure preparation followed as that of Gamez (9) with the slight modification to use nymphs *D. maidis* from a infected colony to prepare the viruliferous leafhoppers.

Symtomatology assay. Symptom ratings were taken periodically and a (33) record was taken of the fourth leave 15 days after the TP from each infected plant. Highly susceptible plants were followed closely to account for possible death. A modified scale of symptomatology based on that one of Toler et al., (33) was devised. All ratings were based on a five-point scale where 0-absence of symptoms; 1-presence of chlorotic dots encompasing 0 to 25% of the leaf area; 2=presence of light chlorotic dots covering between 25 to 50% of the leaf area; 3=presence of continued strips along the axis of the leaves, covering between 50 to 75% of the leaf area and 4=presence of intense symptoms with necrosis of the

leaf tissue, especially along margins and tip of the leaves, affecting 75 to 100% of the leaf area. The different grades of the scale are in the Fig. 1.

Tissue samples. Leaf samples were harvested from each accession 15 days after completion of the TP. The leaves from each plant were pooled and evenly divided into two labeled 1.5 ml microcentrifuge tubes, one to be used for the ELISA, the other for the Slot-blot hybridization assay. The tubes were restored at -20°C until assayed (processed).

Antiserum and inmmunoglobulin preparation. MRFV was purified following the procedure of Leon and Gamez (1981). Antiserum against sucrose gradient purified MRFV was produced in (white rabbit). Virus in 1X PBS (phosphate-buttered saline) was injected intramuscularly after cmulsification with Treund;s complete ajuvant following to procedure of Rivera and Gamez (29). The immunogobulin G (IgG) fraction was purified using exchange chromatography column with protein A. The polyclonal antibodies were quantified by the method of Lowry et al., (1951). Alkaline phosphatase (Type VII-S; Sigma Chemical Co., St. Louis, MO) was coupled to purified IgG by the glutaraldehyde method of Avrameas (2).

Double-antibody sandwich ELISA (DAS-ELISA). The DAS-ELISA procedure followed was as described by Clark and Adams (1977). 500 mg of the different samples were homogenized in a total of 2.5 ml of buffer PBSAT pH 7.2 (NaCl 0.137 M, KCl 0.003 M, Na2HPO4 0.01M, KH2P04 0.002 M plus BSA 4% and Tween-20 0.05%). Coating of the IgG was at a 1:500 dilution and the enzyme-conjugated IgG was at a 1:400 dilution. The plates used were ELISA Nunc (Inter Med., Denmark) and the reactions were recorded at 405 nm in a MR700 Dynatech spectrophotometer (Dynatech, UK).

Filter-bound hybridization (SLOT-BLOT). The slot-blot hybridization procedure was performed in a manner similar to that described by Kafatos *et al.*, (1979). RT-PCR and RNA MRFV was PCR retrotranscribed using the Gene AmpThermostable rTth reverse transcription kit. Perkin Elmer Cetus, CT, USA with specific primers for the capsid protein (27). The fragment about 700 bp obtained was eluted from an agarose gel and labelled with digozygenin following the instructions of the Genius 3 protocol provided with the Nonradioactive DNA labelling and detection kit, (Boerhinger Manheim, RFA) and used as a probe for the hybridization analysis. Samples of total RNA from the different genotypes were prepared according Verwoerd et al., (1989) and spotted on nylon filters (Nytran, Schleicher and Schuell, Keene, N.H.) which had been prewet with ddH_2O and 10X SSC (1XSSC- 0.14M NaCl, 0.014M sodium citrate, pH 7.0) using a slot-blot apparatus (Bio Rad, CA, USA). Filters were crosslinked in the Ultra violet light stratalinker (Strategene Cloning Systems, La Jolla, CA, USA). Prehybridization was performed with a buffer consisting of 5X SC, 0.I% N-laurylsarcosyl, 0.02% SDS, 1% blocking reactive from Genius 3 protocol at 50°C by 6 hrs. For the hybridization the cDNA probe was denatured at 95°C by 5 min an then added to the prehybridization solution (1 ul probe per ml of solution), followed by incubation overnight at 50°C. The filters were washed twice at room temperature with 2X SSC and 0.1% SDS for 15 min followed by two washes with 0.1X SSC and 0.1% SDS at 68°C for 15 min. The

detection and visualization of the hybridization were made using the protocol recommended by LumiPhos ™30.

Data analysis. Statistical analysis were performed at the CIBCM, Costa Rica and repeated at USDA/ARS, Beltsville, Maryland, USA using statistical packages as Minitab (PC-DOS compatible), Geno SAS (PCSAS version 6.04), Statgraphic (PC-DOS compatible), y Statview (MacIntosh). ANOVA, Least statistical differences (LSD), Tukey and linear regression analysis were performed to analyze the response of the different genotypes to MRFV infection (31).

Results

Symptomatology distribution, capsid protein and MRFV RNA detection during the first evaluation.

Symptomatology distribution in different maize genotypes.

The symptoms scale devised is shown in Fig 1. As seen five different categories were established. Most of the genotypes developed symptoms within 2 to 3 days after the completion of the transmission period (TP). Symptoms varied according to the genotype and ranged from moderate to severe (grades 1 to 4). Genotypes 3971 and 2980-93 unlike the others, showed symptoms only 10 to 12 days after completion of the transmission period and they were moderate.

As for individual plants, symptoms started to develop on the fourth leaf spreading out afterwards to the rest of the plant (leaves 5-8), except in some cases in which they appeared on the third leaf. The symptoms usually were over the full leaf but more severe on the proximal region. This effect was more evident in the younger leaves 7 to 8.

The response of the 20 genotypes tested to viral infection was very variable among them as well as among replications and plants within of the different genotypes. The high standard deviation values observed result from considering two different categories within the group exposed plants: a) symptomatic and b) asymptomatic. The ANOVA (Factorail ANOVA) for this data showed significant differences in the responses of the different genotypes to MRFV infection. The LSD analysis at a significance level of a=0.05) yielded various groups of genotypes considering the group of the healthy plants as the control (Table 2).

Genotypes HC-78, 5393 and Guarare showed the highest grades of infection and can be considered as highly susceptible to MRFV (Table 2). This can be clearly observed on analysing the infected-plant distribution since approximately 20 to 30% of the plants showed symptoms reaching grade 4 in the scale (Fig 3). Nonotheless, about 10% of the plants of these genotypes were non-symptomatic, probably due to a failure in the transmission of the virus by the vector. In general between 10 to 20% of non symptomatic plants were observed in the different genotypes (Figs. 3,4,5 and 6).

The genotypes presented in Figs 4 and 5 showed a higher percentage of infected plants with grades of infection 2 and 3 and can therefore be considered as moderately susceptible to MRFV infection. The response of genotypes HC-43 and B-833 to the viral infection is rather interesting since they are commercial

cultivars obtained from the National Production Council (CNP-Costa Rica). In particular the case of HC-43 that 50 and 20% of the infected plants showed symptoms to the grades 3 and 2 respectively. In a like manner, values about of 30 and 40% of infected plants with symptoms to the grades 2 and 3 respectively were determined for the genotype B-833.

The response of genotypes RPM-89, 3175, 3974 and 2980-93 to the infection by MRFV is shown in Fig 6. From this figure, it can be seen that about 50% of the plants in genotypes 3974 and 2980-93 showed no symptoms at all and that only 10 to 20% of the plants showed symptoms in the categories 2 and 3. These symptoms generally appeared 10 to 12 days after completion of the transmission period. It is important, nonetheless to make clear that within the 50% or plants that showed no symptoms, there maybe 10 to 20% of asymptomatic plants resulting from a failed virus-transmission by the vector.

Detection of the MRFV capsid protein by DAS-ELISA

Determination of the DAS-ELISA sensitivity

The sensitivity of the DAS-ELISA test was assayed by using 1:400 IgG dilutions (1 mg mL 1) as antibody bound to microplates and 1:500 of the IGG-Alkaline Phosphatase conjugate (1mg ml-1); both of which were proved to be optimal in previous analyses. To rank a sample as positive the average healthy plant plus three standard deviations criteria was applied, according to Rivera, 1981). The detection threshold was 1 ng of MRFV capsid protein (Fig 7). In comparison extracts from healthy maize plants mixed with MRFV capside protein (to the pure antigen) diluted in PBSAT showed small differences in the absorbance values at 405 nm. It was therefore decided to use absorbance values as detected for healthy maize plant extracts plus the viral capsid protein (Extract MRFV CP), since they better reflect the real concentration in the samples. It was then possible to set up a calibration curve, which stood linear between 1 and 40 ng of viral capsid protein (Fig 7).

To estimate the absolute value of the MRFV capsid-protein in the different genotypes, a formula derived from a correlation between the capsid protein concentration and absorbance at 405 nm was used (Fig 7).

Determination of the MRFV capsid protein using DAS-ELISA in different maize genotypes.

The response of different maize genotypes to viral infection as determined by DAS-ELISA is greatly variabl and so it is for individuals within each genotype. Genotype Guarare on the other hand, expressed the highest capsid-protein level, which fully correlated to the symptomatology it showed (average 2.60 + 1.12). As for the response of genetypes 3974 and 2980-93, nonetheless such a correlation was found since, although they showed a low grade in symptomatology they had capsid protein concentrations similar to those of genotypes moderately susceptible (Table 2).

The genotype 2980-93 instead, had low capsid-protein levels, being one order of magnitude lower than those found in genotypes 2435 and Guarare. Despite of the gradual response observed,

there were no correlation between the capsid protein levels to symptomatology for the other genotypes. Some genotypes such as RPM-89 and 2445 although showed lower values in the symptomatology yielded high concentrations of the capsid protein.

The ANOVA for these data showed significant differences in the response of different maize genotypes to the infection by MVRF. The LSD test at a=o.05, allowed the separation of the genotypes into several groups; the healthy plants considered as the control group.

Determination of viral RNA by molecular hybridization in different maize genotypes inoculated with MRFV.

Total RNA samples were (deposited) on the membranes and hybridized with the labelled probe and developed with a Lumiphos ™530 and/or the precipitable coloration reagents (NBT and BCIP), according to Genius protocol 3. As for Lumiphos ™530 the optimal developing time was 2 hours. When up to 3 hours exposure was performed, the background increased considerably. Using the prcipitable coloration kit, the developing time ranged from 12 up to 18 hours. Longer exposure times brought abcut a full change in the color of the filter. Fig 8 shows a filter developed with Lumiphos ™530. The samples with their respective dilutions as well as the positive (cDNA 1.65 RNA MRFV and viral RNA) and negative (total RNA from non-infected plants) controls can be seen in this figure.

The sensitivity of detection was one ng of viral RNA for both protocols, it was not possible to reduce this level of detection despite the fact that different viral RNA samples, probes, and filter tapes were used as well as the stringency conditions modified.

The response of the different genotypes tested for viral infection was greatly variable, as it was regarding symptomatology and the detection of the capsid protein as determined by DAS-ELISA. The ANOVA for these data showed that there are no significant differences in the responses of the different genotypes tested to the infection by MRFV (Table 2). By using this methodology, it was possible to detect viral RNA in plants showing neither symptoms nor sensible levels of capsid-protein. The viral RNA levels found were similar to those found in plants showing symptoms and capsid-protein. On the other hand, when plants showed symptoms and detectable levels of capsid protein, viral RNA was usually detected.

Linear correlation analysis for symptomatology vs. capsid-protein concentration and viral RNA concentration.

When a linear regression analysis for symptomatology vs. capsid-protein concentration as determined by DAS-ELISA and viral RNA concentration as determined by molecular hybridization was performed, no correlation was found between symptomatology and capsid protein concentration (r=0.18), between symptomatology and viral RNA concentration (r=0.20), nor between the capsid protein concentration and viral RNA (r=0.46) in the different genotypes tested.

Symptomatology distribution and detection of the capsid-protein and viral RNA in the second phase.

In Fig 2 and Table 4 a summary of the interactions occurring during this second phase is presented. As shown, only 40% of the 30 groups of transmitting insects was accountable for the presence of symptoms in genotypes Guarare and HC78 during the first and the third transmission periods. It can be also seen in this table that 13.3% of the transmitting groups was responsible for the appearance of symptoms in genotypes 3974 and 2980-93, although these were mild and delayed in time, as was observed when more detailed analyses were performed.

On the other hand, it was possible to establish that the percentage of plants of genotypes 2980-93 and 3974 showing symptoms in the second transmission period (TP) was significantly lower (36.67%) as compared to those obtained for genotypes Guarare and HC-78 during the first and third; 86.67 and 63.33% respectively (data no showed).

Values of 86.7% and 63.3% in virus transmission in the first and third TP respectively, are significantly higher from the 30% (Gamez, 1980). These data showed that approximately 85% of the plants in the genotypes 3974 and 2980-93 (2nd TP) received viral inoculum and that the attenuated symptomatology observed was not due to a failure virus transmission by the insect.

Symptom distribution in "susceptible" and "resistant" genotypes.

The genotypes symptoms and HC-78 displayed symptoms 2 to 3 days after completion of the transmission period, being generally severe. When plants of genotypes 3974 and 2980-93 showed symptoms, these appeared late (10-15 days after of the TP) and were mild, although in terms of the statistics shown in Table 5, only the plants corresponding to the groups of insects inducing symptomatic plants in the first and third TP but asymptomatic plants in the second TP were considered (Fig 2. Table 4).

Upon evaluating symptomatology it was found that genotype HC-78 showed the highest grade of symptoms with a mean of 3.75 which implies intense symptoms; about 75% of the leaf area were affected. The genotype Guarare has a mean of 2.75 which represents about 50% of the leaf area with symptoms (see Figs 1 and 9). The values for HC-78 and Guarare in this phase are higher than the 2.88 and 2.60 determined respectively in the first phase (Tables 2 and 4). The high standard deviation values observed confirm the great variability existing among plants in both genotypes.

The ANOVA analysis for these data showed the presence of significant differences between susceptible and resistant genotypes to MRFV infection. Tukey's multiple comparisons test at a significance level of u=0.01 allowed the separation of genotypes into two groups, being the resistant genotypes comparable to the group of healthy plants (Table 4).

Determination of the capsid-protein by DAS-ELISA in "resistant" and "susceptible" genotypes.

The response to virus infection at the different maize genotypes in the 2nd phase as determined by DAS-ELISA was highest variable as it was in the first phase. It was possible to establish that the susceptible genotypes HC-78 and Guarare express very high levels of viral capsid-protein (336.10 and values are in accordance with the symptomatology shown by those genotypes but different from 280.35 and 347.15 ng MRFV-CP/mg tissue measured during the first phase (Table 158.20 of ng MRFV-CP/mg fresh tissue respectively). These2).

On the other hand, the "resistant" genotypes 2980-93 and 3974 showed very low levels of virus capsid protein (14.1 and 0.39 ng MRFV-CP/mg fresh tissue respectively). The concentration corresponding to genotype 2980-93 is very similar to that found it in the first phase 17.3 ng MRFV-CP/mg fresh tissue, but for genotype 3971 it was significantly different from that found in the first phase (144.61 ng CP-MRFV/mg tissue, Table 2). The ANOVA for these data showed significant differences between susceptible and resistant genotypes to MRFV infection as determined by capsid-protein concentration. Tukey's multiple comparisons test at a significance level of (a=0.01) allowed the separation of the genotypes into two groups, placing the resistant ones in the same category as healthy plants (Table 4).

MRFV-nucleic acids detection in the "resistant" and "susceptible" genotypes.

The levels of viral RNA found either in "susceptible" and "resistant" genotypes were similar. The viral RNA concentration in the susceptible genotypes as 0.39 and 0.16 ng MRFV-RNA/mg tissue for Guarare and HC-78 respectively (Table 4). The value found for genotype IIC-78 was similar to that obtained during the first phase (0.15 ng MRFV-RNA/mg tissue) but different in the genotype Guarare since 0.14 ng MRFV-RNA/mg tissue, were measured during the first phase (Tables 2 and 4). Regarding the values found for the resistant genotypes 2980-93 and 3974, 0.39 and 0.27 ng MRFV-RNA/mg tissue respectively (Table 4), were similar or even higher than those found in the susceptible materials. They are also different from those obtained during the first phase (Table 2). The ANOVA values obtained from these data showed that there are no significant differences in the response of the different genotype to MRFV infection.

Linear correlation analysis for symptomatology, capsid-protein and MRFV genomic RNA concentration in "susceptible" and "resistant" genotypes.

When linear regression analysis were performed between symptomatology vs. capsid-protein concentration determined by DAS-ELISA and symptomatology vs viral RNA concentration as determined by SLOT-BLOT, a correlation between symptomatology and capsid-protein was found (r-0.8) which contrasts with the results obtained in the first phase when no correlations were found (r=0.2). Nonetheless no correlation were found it neither for symptomatology vs viral RNA concentration nor capsid-protein

concentration vs viral RNA levels.

Conclusions:

Acknowledgements:

This work was supported by the USAID/PSTC grant number 936-5542.13, of the project number 9.198 and by The Latin American Plant Science Network (RLB). We would like to acknowledge to M.Sc. Federico Albertazzi for their technical assistance and valuable discussions.

P.I.B. acknowledge the M.Sc. Fellowship given for the RLB.

Literature cited:

1. Ancalmo, O. & W. C. Davis (1961). Achaparramiento (corn stunt). Plant Dis. Reptr. 45:281.
2. Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry 6:43-52.
3. Bretting, P. K., M. M. Goodman & C. W. Stuber. (1987). Karyological and isozime variation in West Indian and allied American mainland races of maize. Amer. J. Bot. 74:1601-1613.
4. Clark, M. F. & A. N. Adams. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
5. Davis, R. E & J. F. Worley. (1973). Spiroplasma: Motile, helical, microorganism associated with corn stunt disease. Phytopathology. 63:403-408.
6. Espinoza, A. M., P. Ramirez & P. Leon. (1988). Cell-free translation of maize rayado fino virus genomic RNA. J. Gen. Virol. 69:757-762.
7. Fraser, (1990).....
8. Gamez, R. (1969). A new leafhopper borne of corn in Central America. Plant Dis. Reptr. 53:929-932.
9. Gamez, R. (1973). Transmission of rayado fino virus of maize (**Zea mays L.**) by **Dalbulus maidis**. Ann. Appl. Biol. 73:285-292.
10. Gamez, R. (1980). Maize Ravado Fino Virus. Descriptions of Plant Viruses. C.M.I./A.A.B. 220. 4pp.
11. Gamez, R. (1983). The ecology of maize rayado fino virus in the American Tropics. In: Plant Virus Epidemiology (R. T. Plumb and J. M. Tresh, eds.) 267-275 pp. Blackwel Scientific, Oxford, England.
12. Gamez, R. & P. Leon. (1983). Maize rayado fino virus: Evolution with plant host and insect vector. In: Current Topics in Vector Research. (K. F. Harris., ed). 149-168 pp. Praeget Scientific, N. York.
13. Gamez, R. & P. Leon. (1985). Ecology and evolution of a neotropical leafhopper-virus-maize association. In: The Leafhopper and Planthoppers. (L. R. Nault and J. G. Rodriguez, eds.), 331-350 pp. J. Whiley & Sons, Inc., N. York.
14. Gamez, R. & P. Leon. (1988). Maize Rayado Fino and Related Viruses. In: The Plant Viruses. Vol III (R. Koening, ed), 213-233 pp.
15. Itis, H. H. (1983). From teosinte to maize:The catastrophic sexual transmission. Science 222:886-894.

16. Kafatos, F. W., C. W. Jones & A. Efstradiadis. (1979). Determination of nucleic acid sequence homologies and relaltive concentrations by dot hybridization procedure. Nucelic Acid Res. 7:1541-1553.
17. Kim, S. K., Y. Efron, I. M. Fajemisin, & I. W. Buddenhagen. (1989). Mode of gene action for resistance in maize to maize streak virus. Crop Sci. 29:890-894.
18.Leon, P. & R. Gamez. (1981). Some physicochemical properties of maize rayado fino virus. J. Gen. Virol. 56:67:75.
19. Lockhart, B. E., N. Khales, A. N. Lennon, & N. el Meatauoi. (1985). Properties of Bermuda grass-etched-line virus a new leafhopper transmitted virus related to maize rayado fino and oat blue dwarf viruses. Phytopathology 75:1258.
20. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265.
21. Mangelsdorf, P. C. (1974). Corn, its origin, evolution and improvement. Cambridge: Harvard University press. 262 pp.
22. Maule, A. J., R. Hull, & J. Donson. (1983). The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. I. Virol methods 6:215-224.
23. Moseley & Hull, R. (199).
24. Nault, L. R. & E. D. Ammar. (1989). Leafhopper and Planthopper transmission of plant viruses. Ann. Rev. Entomol. 34:503-529.
25. Nault, L. R. R. E. Gingery & D. T. Gordon. (1980). Leafhopper transmission and host range of maize rayado fino virus. Phytopathology 70:709-712.
26. Owens, R. A. & T. O. Diener. (1984). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.
27. Ramirez, P. & R. Hammond. (1987). Cloning of a DNA copy of maize rayado fino virus (MRFV). International Congress of Plant Tissue Culture. Tropical species. Bogota-Colombia.
28. Rivera, C. & R. Gamez. (1983). Adaptacion de una tecnica de inmunosorbencia (ELISA) a la deteccion del virus del rayado fino del maiz en su insecto vector. Turrialba 33:177-184.
29. Rivera, C. & R. Gamez. (1986). Multiplication of maize rayado fino virus in its leafhopper vector *Dalbulus maidis*. Intervirology. 25:76-82.
30. Rocha-Pena, O. (1981). Algunos aspectos relacionados con el virus del rayado fino del maiz en Mexico. Tesis, M.Sc. Colegio de Posgraduados, Chapingo, Mexico.
31. Steel, R. & T. Torrie. (1985). Bioestadistica Principios y procedimientos. 2 Ed. McGraw Hill, Edit. Colombia 622 p.
32. Stoddard
33. Toler, R. W., G. Skinner, A. J. Bockholt & K. Harris. (1985). Reactions of maize (*Zea mays*) accesions to maize rayado fino virus. Plant Dis. 69:56-57.
34. Verwoerd et al. (1989).
35. Vloten-Doting, L. (1989). Symptoms of plant disease mediated by plant virus gene products. In: Molecular Bases of Virus diseases. pp 303-318.
36. Zaitlin, M. & R. Hull. (1987). Plant virus-host

interactions. Ann Rev. Plant Physiol. 38:291-315.

Table 1.
Different genotypes of maize screened for MRFV resistance.

Accesion No.	Race	Source
9063	Caniil9	CIMMYT
9823	Caniil9	"
5393	Chande9	"
9053	Chande9	"
3175	Comun9	"
3190	Costen9	"
3195	Costen9	"
9887	Cubaam9	"
2445	Ftcuba9	"
3201	Puya9	"
2490	Tuson9	"
3974	StCroi	"
5431	Tuxpen	"
2435	Criol1	"
2980-93	Hybrid	"
RPM-89	Hybrid	EEFB
Guarare	Hybrid	"
HC-43	cv. comercial	CNP
HC-78	cv. comercial	"
B-833	cv. comercial	"

Table 2.
Effect of the MRFV infection on different maize genotypes as determined by symptomatology, ELISA and SLOT-BLOT hybridization during the first evaluation.

Genotypes	Symptomatology*	ELISA** (ng of CP-MRFV/mg tissue)	SLOT-BLOT hybridization** (ng of viral RNA/mg tissue)
HC-78	2.88 ±1.05a***1	280.35 ± 200.85 abc***1	0.15 ± 0.20 a***2
5393	2.64 ±1.49ab	118.27 ± 116.51 bc	0.14 ± 0.19 a
Guarare	2.60 ±1.12ab	347.15 ± 299.56 ab	0.14 ± 0.20 a
9823	2.44 ±1.04 bc	272.39 ± 170.17 abc	0.20 ± 0.22 a
9063	2.44 ±1.38 bc	177.23 ± 179.84 abc	0.30 ± 0.24 a
HC-43	2.28 ±1.27 bcd	144.60 ± 126.93 bc	0.03 ± 0.02 a
5431	2.28 ±1.27 bcd	325.86 ± 295.20 ab	0.19 ± 0.23 a
3190	2.28 ±1.14 bcd	277.92 ± 193.04 abc	0.19 ± 0.23 a
9887	2.16 ±1.25 cde	196.87 ± 193.92 abc	0.24 ± 0.25 a
3195	2.12 ±1.01 cdef	244.78 ± 286.66 abc	0.29 ± 0.24 a
2490	2.12 ±1.05 cdef	166.81 ± 179.84 abc	0.09 ± 0.16 a
9053	2.08 ±1.15 cdef	286.17 ± 261.33 abc	0.25 ± 0.24 a
B-833	1.96 ±1.24 def	152.84 ± 181.67 bc	0.08 ± 0.16 a
3201	1.88 ±1.16 efg	253.98 ± 211.27 abc	0.14 ± 0.20 a
2445	1.88 ±1.30 efg	280.96 ± 338.38 abc	0.19 ± 0.22 a
2435	1.84 ±1.06 efg	432.45 ± 692.14 a	0.32 ± 0.37 a
RPM-89	1.76 ±1.16 fg	324.86 ± 272.51 ab	0.09 ± 0.15 a
3175	1.56 ±1.04 g	104.93 ± 107.92 abc	0.03 ± 0.02 a
3974	0.88 ±1.09 h	144.60 ± 284.82 bc	0.13 ± 0.21 a
2980-93	0.48 ±0.65 i	17.30 ± 51.05 c	0.08 ± 0.15 a
Healthy plant	0.00 ±0.00 j	0.02 ± 0.01 d	0.00 ± 0.00 a

* average from five replication and considering five plants by each replication
** average from three replication and considering three plants by each replication
***1 according LSD's test at significance level of α = 0.05.
***2 according Tukey's test at significance level of α = 0.05.

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Table 3.
Transmission efficiency of *D. maidis* groups in the second evaluation of "resistant" and "susceptible' genotypes of maize.

1st TP (n=30 plants)	2nd TP (n=30 plants)	3rd TP (n=30 plants)	N° of groups	%
+	+	+	4	13.3
+	+	-	5	16.6
+	-	+	12	40.0
+	-	-	2	6.7
-	+	+	2	6.7
	-	-	2	6.7
-	+	.	0	-
-	-	+	0	-
ND	ND	ND	3	10.0
TOTAL			30	100.0

ND= non determinated.
*: 10-12 *D.maidis* /group.
+ = groups of *D.maidis* with symptomatic plants.
- = groups of *D.maidis* with non-symptomatic plants.

Table 4.
Effect of MRFV on "susceptible" and "resistant" genotypes of maize as determined by symptomatology, ELISA and SLOT-Hybridization in the second evaluation.

Genotypes*1	Symptomatology*2	DAS-ELISA [ng of CP-MRFV/mg tissue]	SLOT-BLOT hybridization [ng of viral RNA/mg tissue]
HC-78	3.75 ± 0.50 a*3	336.10 ± 100.90 a*3	0.16 ± 0.20 a*3
Guarare	2.75 ± 1.26 a	158.20 ± 100.40 a	0.39 ± 0.22 a
2980 93	0.00 ± 0.00 b	14.10 ± 27.70 b	0.39 ± 0.22 a
3974	0.00 ± 0.00 b	0.39 ± 0.52 b	0.28 ± 0.25 a
healthy plant	0.00 ± 0.00 b	0.04 ± 0.02 b	0.00 ± 0.00 b

*1: average of 4 plants
*2: 0= no symptoms...4= severe symptoms
*3: according to Tukey's test, different letters correspond to statistical different group at significance of α= 0.01.

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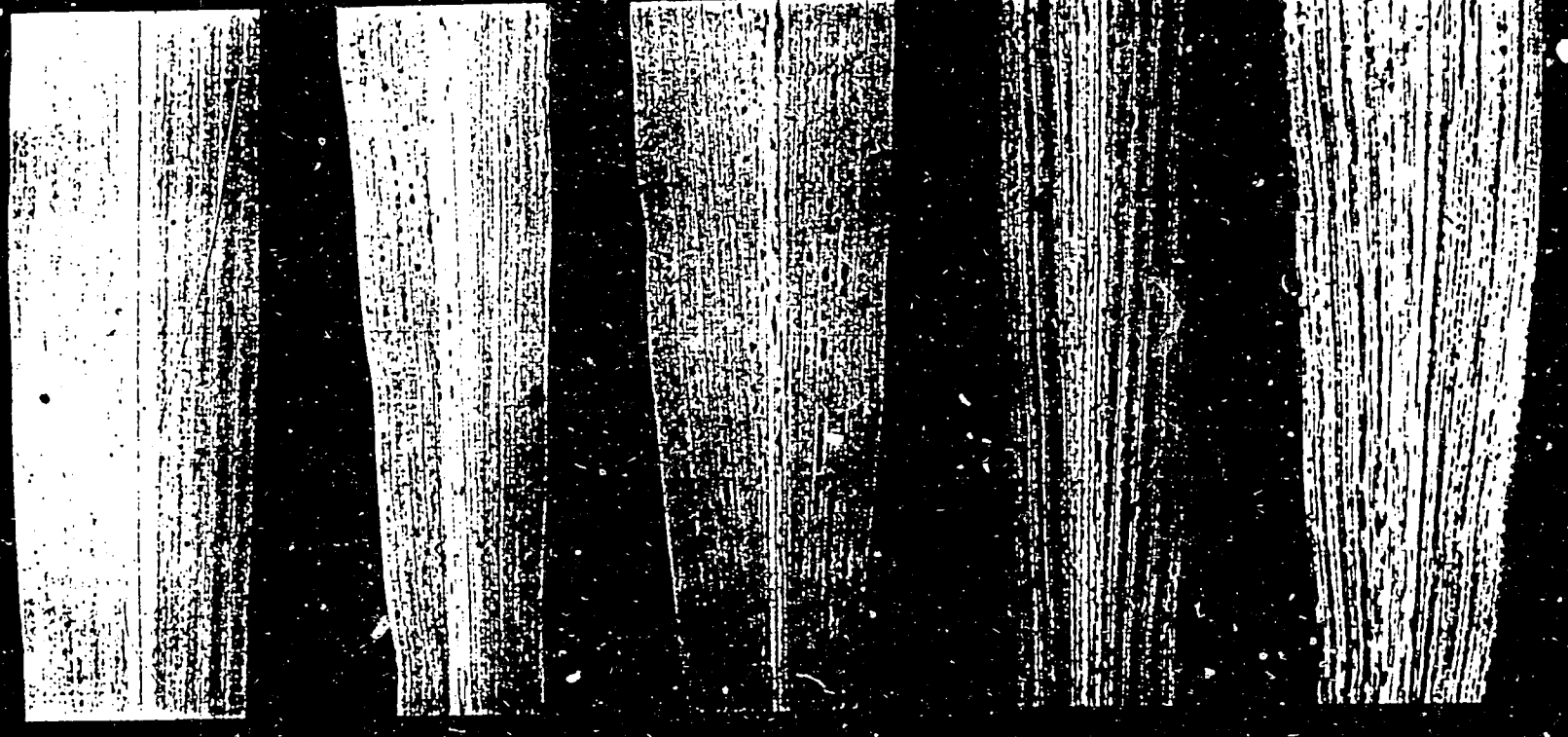
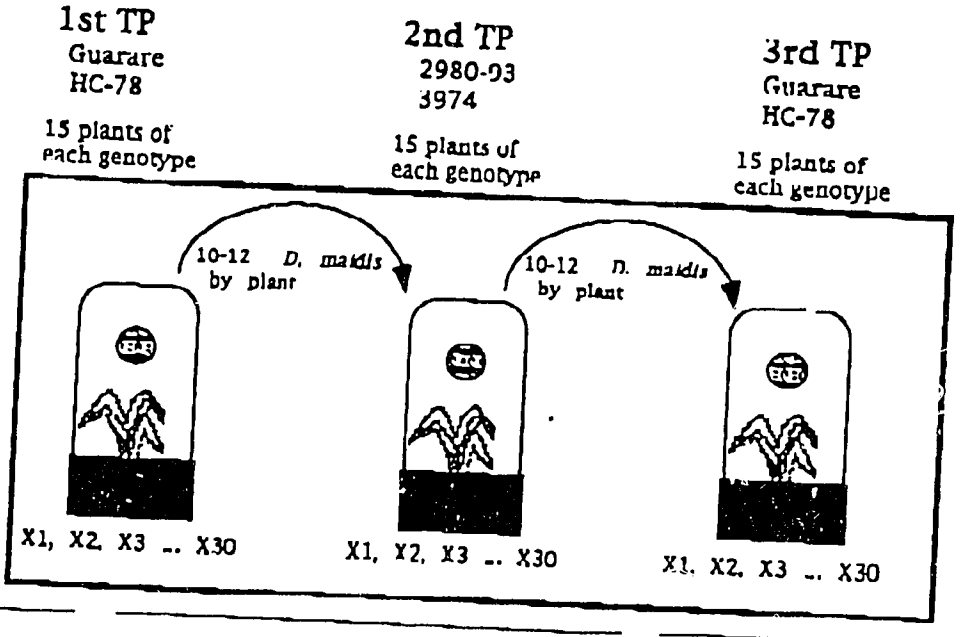


Escala de síntomas utilizada para evaluar la respuesta de los diferentes genotipos de maíz a la infección del VRFM.

Fig 2. Schematic representation of the inoculation and symptoms distribution on the "susceptible" and "resistant" genotypes to MRFV infection in the second evaluation.



Group of insect	"susceptible" Genotypes	N° of plant	"resistant" Genotypes	N° of plant	"susceptible" Genotypes	N° of plant
X1	Guarare (S)	1001	2980-93 (S)	1061	HC-78(S)	1121
X2	Guarare (S)	1002	2980-93(NS)	1062	HC-78(S)	1122
X3	Guarare (S)	1003	3974 (S)	1063	HC-78(NS)?	1123
X4	Guarare (NS)	1004	3974(S)†	1064	HC-78(S)	1124
X5	HC-78 (S)	1005*	2980-93 (ND)	1065*	Guarare (ND)	1125
X6	HC-78 (S)	1006	3974 (S)	1066	HC-78(S)	1126
X7	HC-78 (S)	1007	3974 (S)	1067	HC 78(S)	1127
X8	Guarare (S)	1008	3974†(ND)	1068	Guarare(S)	1128
X9	Guarare (NS	1009	3974 (S)	1069	HC-78(S)	1129
X10	HC-78 (S)	1010	2980-93(NS)	1070	Guarare(S)	1130
X11	HC-78 (S)	1011	3974(NS)†	1071	Guarare(S)	1131
X12	HC-78 (S)	1012	2980-93(NS)†	1072	Guarare(S)	1132
X13	HC-78 (S)	1013	3974(NS)†	1073	HC-78(S)	1133
X14	HC-78 (NS)	1014	2980-93(NS)	1074	HC-78 (NS)	1134
X15	Guarare (S)	1015	3974(NS)	1075	Guarare(S)	1135
X16	HC-78 (S)	1016	3974(NS)	1076	HC-78 (NS)	1136
X17	Guarare (S)†	1017	2980-93 (S)	1077	Guarare (NS)	1137
X18	HC-78 (S)	1018	2980-93(NS)	1078	Guarare(S)	1138
X19	Guarare (S)	1019	3974 (ND)†	1079	HC-78 (NS)	1139
X20	Guarare (S)	1020	3974 (S)	1080	Guarare (NS)	1140
X21	Guarare (NS)	1021	2980-93(NS)	1081	Guarare(NS)	1141
X22	HC-78 (S)	1022	2980-93 (S)	1082	Guarare(NS)	1142
X23	HC-78 (S)	1023	3974(NS)	1083	Guarare (S)	1143
X24	Guarare (S)	1024	2980-93(NS)	1084	Guarare (S)	1144
X25	HC-78 (S)	1025	3974 (NS)	1085	HC-78 (S)	1145
X26	Guarare (S)	1026	2980-93 (NS)	1086	Guarare(NS)	1146
X27	Guarare (S)†	1027	2980 93 (S)	1087	HC-78 (NS)	1147
X28	Guarare (S)	1028	2980-93(S)	1088	HC-78 (S)	1148
X29	HC-78 (S)	1029	2980-93 (NS)	1089	Guarare (S)	1149
X30	HC-78 (S)	1030	3974 (NS)	1090	HC-78 (S)	1150

† – dead plant
* =dead insects

(S) – Symptomatic plant
(NS)– Non-symptomatic plants

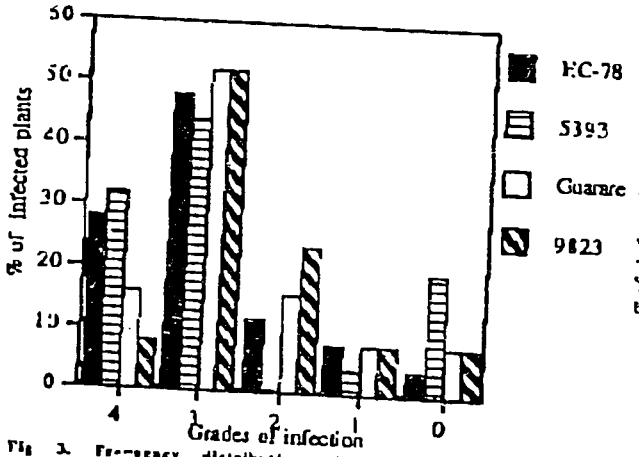


Fig 3. Frequency distribution plot as percentages of infected plants according to the grades of infection on genotypes HC-78, 5393, Guanare and 9823

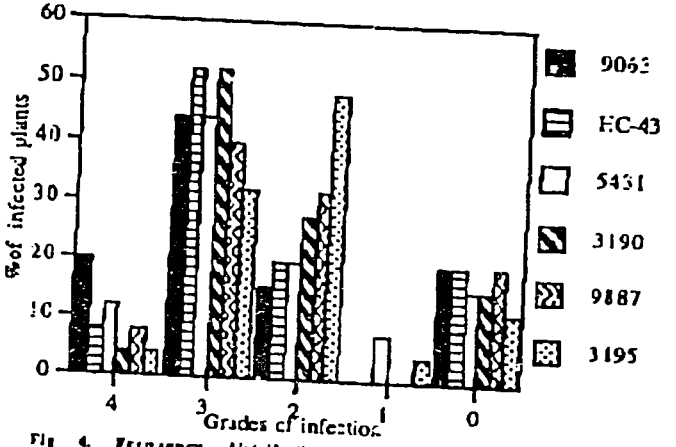


Fig 4. Frequency distribution plot as percentages of infected plants according to the grades of infection on genotypes 9063, HC-43, 5431, 3190, 9887 and 3195

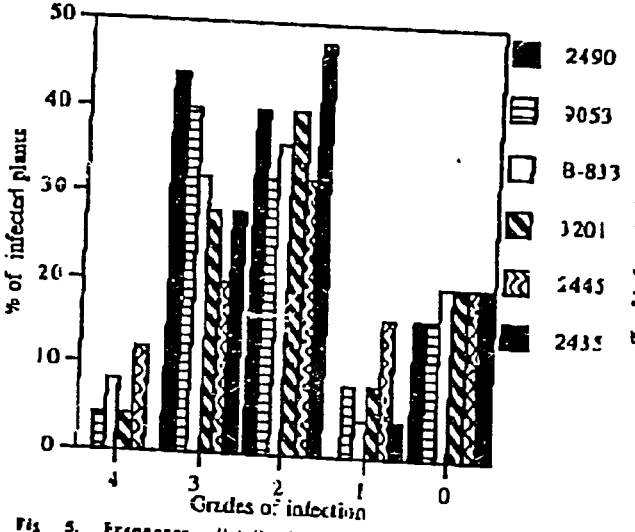


Fig 5. Frequency distribution plot as percentages of infected plants according to the grades of infection on genotypes 2490, 9053 B-833, 3201, 2445 and 2435.

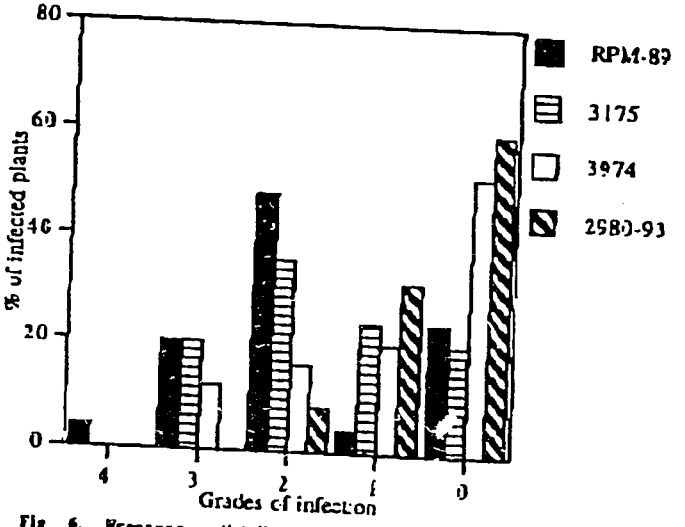


Fig 6. Frequency distribution plot as percentages of infected plants according to the grades of infection on genotypes RPM-89, 3175, 3974 and 2980-93.

Appendix III

Pre-proposal FY93

Future Plans

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AID - PSTC Pre-Proposal

University of Costa Rica - USDA-ARS

November 5, 1992

1. Title: Genome Organization and Expression Strategy of Maize Rayado Fino Virus

2. Investigators:

Dr. Pilar Ramirez
Principal Investigator
Plant Virologist
CIBCM and School of Biology
University of Costa Rica
San Jose, Costa Rica
Telephone 506-535323, ext 5721
FAX 506-249367

Dr. Rosemarie Hammond
US Cooperating Investigator
Research Plant Pathologist
Microbiology and Plant Pathology Lab
USDA/ARS Beltsville, Maryland
20705
Telephone 301-504-5203
FAX 301-504-5449

Dr. Marta Valdez
Collaborator
School of Biology
University of Costa Rica
San Jose, Costa Rica

Submitting Institution: U S Department of Agriculture - Agricultural Research Service

Project Duration: 3 years
Requested Funding: $ 149,600

3. Project Objectives:

The *scientific* objective of our project is to determine the strategy of genome expression of maize rayado fino virus (MRFV) in its insect and plant hosts and to apply this knowledge towards improvement and/or introduction of resistance in maize to this important pathogen. The ultimate objective is to enable the LDC scientists to obtain the advanced tools needed to investigate the molecular biology of plant viruses in order to gain valuable knowledge necessary for designing disease control strategies.

4. Technical Discussion:

The leafhopper-borne maize rayado fino virus (MRFV) is the only known indigenous virus of maize in Mesoamerica and it is of great agronomic importance as it produces significant losses in maize throughout Central and South America (Gamez and Leon, 1983). In the field, MRFV is usually associated in a disease complex with corn stunt spiroplasma and maize bushy stunt mycoplasma which are both transmitted by the same vector as MRFV. No resistant maize cultivars are known, although some landraces express some level of tolerance. An understanding of the life cycle of the virus is necessary to develop novel control strategies for the disease.

MRFV is the best characterized member of the marafivirus group. It possesses small isometric particles, a single stranded RNA genome of 2.0 - 2.1 x 10^6 Mr which lacks a 5' VPg and 3' poly A tail (Gamez and Leon, 1987). The viral capsid is composed to two serologically related proteins of 22kd and 28kd molecular weight. In reticulolysate lysates, the MRFV genomic RNA directs the synthesis of proteins ranging in size from 15 to 165 kd, however, no polypeptide corresponding to the capsid protein genes could be detected by immunoprecipitation of the translation products with antisera to the capsid protein (Espinoza et al., 1988). The strategy of expression of the MRFV genome and its capsid protein are therefore unknown, although the virus may produce a single subgenomic RNA of 1.1 kb in infected plants from which the capsid protein may possibly be translated (Karkashian and Hammond, unpublished results). In many respects, MRFV differs from most well characterized plant RNA viruses in the combination of properties of its virion RNA, coat protein expression, and biological characteristics.

There is a close association between MRFV, its insect vector Dalbulus maidis, and its maize host. Both virus and vector have narrow and overlapping host ranges. In addition, MRFV replicates in both its insect and plant hosts. As such, the marafiviruses are the smallest viruses known to replicate in both insect and plant hosts, and it is not known which factors affect multiplication and transmission. Our scientific hypothesis is that essential regulatory information for the maturation and translation of the virus in both cell types exists in the sequence of its genome.

Experimental Design:

1) *Determine the genomic organization of MRFV.*

Viruses with single-stranded RNA genomes exhibit great variety in genome structure and expression (Matthews, 1991). MRFV has a monopartite genome and in preliminary work towards determining its expression, we have generated cDNA clones corresponding to a significant proportion of the genome. We have determined the coding region of the MRFV capsid protein by sequence analysis and in vitro expression of fusion proteins in E. coli. In order to complete our knowledge of the genomic organization of MRFV, we plan to obtain the complete sequence of MRFV and to compare sequences of potential protein open reading frames (ORFs) with other known RNA viral proteins, specifically nonstructural proteins such as the replicase for which identifying sequence motifs exist. This is an approach that has been widely used for mapping functional ORFs in plant viral genomes (eg. Miller et al., 1988). The genomic organization will also be determined by cistron mapping using hybrid-arrest translation of the genomic RNA in wheat germ and reticulocyte lysate in the presence of genome-derived cDNA fragments and to identify translated proteins using antisera prepared as in #2 (Hellman et al., 1986).

An ultimate goal is the construction of a full-length cDNA clone of MRFV from which infectious transcripts can be synthesized for use in examining and manipulating expression of the genome in plant and insect cells in order to identify potential regulatory signals (see # 3 below) (Bujarski and Miller, 1992). It is also advantageous to obtain a full-length cDNA because the virus is difficult to manipulate by vector-infection. An infectious cDNA would allow particle bombardment of plant tissue to reduce reliance upon the insect vector for virus maintenance and protoplast studies. Additional cDNA cloning will be necessary to construct the full-length cDNA - most likely by utilizing sequence specific primers from the 5' terminus to assemble the genome (Weiland and Dreher, 1989).

2) *Prepare antisera to ORFs for use in study of gene expression in plant and insect cells.*

In order to study the expression of the genome in vitro and to define ORFs that are actually functional in vivo (see #3 below), we will need to raise antisera to ORFs by using as antigens either synthetic peptides

determined from the translation of the sequence or proteins synthesized by overexpression in E. coli from ORF fragments subcloned into pTrcHis vectors (Invitrogen, San Diego, CA; Studier and Moffatt, 1986).

3) *Study the genome expression strategy of MRFV in plant and insect cells.*

MRFV is a difficult virus to work with because a) it is appears to be confined to the phloem and therefore occurs at relatively low concentrations in the infected plant, and b) it is not transmitted by mechanical inoculation. We have studied the accumulation of MRFV in infected whole plants and have used cloned probes to evaluate selected maize germplasm for susceptibility, tolerance, or resistance to the virus (Bustamante et al., manuscript in preparation). We have also tried to examine MRFV accumulation in whole insects. In this project, we propose to study the replication and expression of the MRFV genome in a) *plant cell protoplasts* b) *insect monolayer cell cultures*.

Plant cell protoplasts have been used extensively for the study of viral gene expression (Harrison and Mayo, 1983). Fuentes (1986) discovered that MRFV genomic RNA would replicate in a protoplasts prepared from a non-host, bean (Phaseolus vulgaris), following PEG co-precipitation of viral RNA. With the assistance of Dr. Valdez, we will repeat these experiments and develop a protoplast system from maize in order to examine replication and expression of the genome in the susceptible and tolerant genotypes of maize mentioned above. Replication will be determined using strand-specific cRNA probes (Albertazzi et al., manuscript in preparation) and expression of the genome will be examined with antisera prepared in #2. Isolation of possible subgenomic RNAs and determination of the expression strategy used for the capsid protein may be more feasible in the protoplast system.

If the virus will replicate in the protoplasts of other species which are easily transformed, eg. members of the Solanaceae, one could envision the introduction of constructs containing the capsid protein or replicase genes in order to observe their effect on the replication of viral RNA introduced into protoplasts derived from the transformants (eg. Lindbo and Dougherty, 1992).

Kimura and Black (1972) and Hsu and Black (1974) showed that synchronous infection of leafhopper transmitted plant viruses in *insect monolayer cell cultures* could be achieved. In the report by Hsu and Black (1974) 100% infection was achieved within 9 hours post-inoculation using a purified virus suspension. Falk et al. (1987) observed a difference in viral genome expression in whole plants and insects infected with the leafhopper-transmitted maize stripe virus. We will examine the similarities and differences in expression of the virus in the insect as compared to the plant cells. Dr. Hsu (USDA, Beltsville) has offered to provide insect cell lines and to help develop specific vector cell lines for MRFV.

5. Innovation:

The proposed research will use state-of-the-art molecular biological and virological methods to determine the heretofore unknown genome expression strategy of the type member of the marafivirus group, the smallest viruses known to replicate in both their plant and insect hosts. The successful completion of this project will considerably improve our understanding of the MRFV infection process and allow us to introduce effective control measures by either introducing sense or antisense MRFV genes into maize or by using resistant or tolerant genotypes to breed into maize lines used in the field.

6. Relevance:

In Costa Rica and many developing countries of Latin America, maize is one of the principal food sources. Although maize is a main source of food in these countries, yields are low, averaging 1.3 metric tons/ha, considerably less than the 3.1 tons/ha world average (CIMMYT, 1981). There is no doubt that maize rayado fino virus contributes to the reduced yield. The incidence of MRFV infection generally varies from 0-40%, but in some areas (Pacific lowlands of El Salvador: highlands of Colombia) it reaches nearly 100% (Gamez et al., 1979). In field infections, MRFV occurs often associated with mollicutes (a spiroplasma and and a mycoplasma). This association is known as the maize stunt complex and is considered by CIMMYT as one of the most common and economically important diseases in Mexico and Central and South America. Our proposed research on MRFV and its interaction with its insect and plant hosts addresses important questions about the infection process which will aid in our goal of introducing resistance to MRFV into maize cultivars grown in this region of the world. In the long-term, once resistant cultivars are obtained, these will be made available to LDC breeders.

7. Building Research Capacity:

This research will complement and enhance a high priority research program at the University of Costa Rica on the study of the cellular and molecular biology of viruses and other pathogens endemic to the tropics and important to man, cultivated plants or domestic animals. It will also complement a second program being developed in the School of Biology (Dr. Marta Valdez) based on developing plant transformation technology with one goal being the introduction into crop plants of resistance to pathogens.

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This project will enable a strengthening and introduction of modern techniques in molecular biology and virology into a laboratory that is skilled in techniques such as insect rearing and viral transmission studies, viral extractions, serology, electron microscopy, and the use of nucleic acid probes for viral detection.

8. Collaboration:

The US and Costa Rican PI have an established, ongoing collaborative effort in the study of MRFV as well as continued and expanding collaborations in other areas of virology. The Costa Rican and US collaborator exchange information, knowledge, technology, and materials. The Costa Rican PI as well as her graduate students have made visits to the US laboratory for the purpose of conducting collaborative research and for training. The US cooperator has travelled to Costa Rica to perform experiments and to train the LDC scientists. This type of collaboration will continue and be enhanced in this project with the training of a Costa Rican M.Sc. student at the University of Maryland Department of Botany Ph.D. program. The graduate student upon completion of the Ph.D. degree would return to the University of Costa Rica to begin a program in virus research there. cDNA cloning, sequence analysis, in vitro translation studies and and insect cell culture studies will be performed at USDA with transfer of the technology to UCR. Plant cell protoplast studies and additional manipulations will be performed at the UCR.

The submitting institution is the USDA and not the University of Costa Rica in order to facilitate management of the project and to avoid any problems that may be encountered with the LDC AID mission. Subcontracting to UCR will be used to transfer funds.

9. Tentative Proposed Budget (In US $):

	Year 1		Year 2		Year 3		Total	
	CR	US	CR	US	CR	US	CR	US
Salaries a		15000		15000		15000		45000
Salaries b		8000		3000		8000		24000
Salaries c	12000		12000		12000		36000	
Supplies/ Equipment[d]	4000	4000	4000	3000	4000	3000	12000	10000
Travel[e]	1500	1500	1500		1500	1500	4500	3000
Training/ Consultation[f]	500		500		500		1500	
Overhead[g]	1800	2850	1800	2600	1800	2750	5400	8200
	19800	31350	19800	28600	19800	30250	59400	90200

Total = $149,600

a Annual stipend and fees for Costa Rican graduate student at the University of Maryland.
b Salary for a part-time student technician for the US cooperator
c Salaries for two students ($6000 each) at the University of Costa Rica, one to work with Dr. Ramirez on molecular aspect of the project and one for Dr. Valdez on the study of genome expression in plant cell protoplasts.
d Expendables such as plastics, reagents, and radioisotopes
e Includes travel and per diem for scientists and students.
f Training for students and PI is included in a and e. Additional money is requested in this section for BITNET.
g 10 % overhead for UCR and USDA

References

Albertazzi, F., Hammond, R. W., Rivera, C., and Ramirez, P. manuscript in preparation.
Bujarski, J. J. and Miller, W. A. 1992. In: Genetic Engineering with Plant Viruses, CRC press, 115-147.
Bustamante, P., Albertazzi, F., Hammond, R., and Ramirez, P. manuscript in preparation.
Espinoza, A. M., Ramirez, P., and Leon, P. 1988. J. gen. Virol., 69, 757-762.
Falk, B. W., Tsai, J. H., and Lommel, S. A. 1987. J. gen. Virol., 68, 1801-1811.
Fuentes, A. L. 1986. M.Sc. Thesis. University of Costa Rica, San Jose, Costa Rica.
Gamez, R. and Leon, P. 1983. In: Current Topics in Pathogen-Vector-Host Research (K. Harris, ed.) Praeger Scientific, New York. pp. 149-168.
Gamez, R. and Leon, P. 1987. In; Plant Viruses. Plenum, New York. pp. 213-223.
Harrison, B. D. and Mayo, M. A. 1983. In: Use of Tissue Culture and Protoplasts in Plant Pathology, Academic Press, Australia. pp. 69-137.
Hellman, G. M., Hiremath, S. T., Shaw, J. G., and Rhoads, R. E. 1986. J. gen. Virol. 151, 159-171.
Hsu, H.- T. and Black, L. M. 1974. Virology 59, 331-334.
Kimura, I. and Black, L. M. 1972. Virology 48, 852-854.
Lindbo, J.A. and Dougherty, W. G. 1992. Virology 189, 725-733.
Matthews, R.E.F. 1991. Plant Virology, Third Edition, Academic Press, San Diego, CA. 835 pp.
Miller, W. A., Waterhouse, P. M., and Gerlach, W. L. 1988. Nucleic Acids Res. 16, 6097-6111.
Weiland, J. J. and Dreher, T. W. 1989. Nucleic Acids Res. 12, 4675-4687.

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