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CENTRO AGRONOMICO TROPICAL
DE INVESTIGACION Y ENSEÑANZA

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May 17, 1993

Dr. Miloslav Rechcigl
Office of Research, AID/R&D/R
Room 320 SA-18
Washington, DC 20523-1818
USA

Re: "Theobroma cacao DNA as a Marker for For Plant Breeding", 7.095

Dear Dr. Rechcigl:

In a letter dated August 24, 1992, Dr. John Daly asked me to submit a final report on the above referenced grant. Many circumstances prevented me from satisfying this request until now and I apologize for any inconvenience this may have caused. I hope that the enclosed final report on this grant will be acceptable at this time. Thank you for your patience and your understanding.

For the record please note that my current address is changed and that I am currently working at CATIE in connection with our currently funded AID grant 10.117 "Theobroma cacao: DNA Analysis for Fungal Resistance".

Sincerely yours,

Paul J. Fritz

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CC: C DIE + BIST ID:

PN-AEI-370

FINAL REPORT

Covering Period July 31, 1987 to August 1, 1990

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

Theobroma cacao DNA as a Marker for Plant Breeding

Principal Investigator: Paul J. Fritz
Grantee Institution: The Pennsylvania State University

Collaborator: Ramon Lastra
Institution: Centro Agronomico Tropical de Investigacion y Enseñanza (CATIE)
Tropical Agriculture Research and Teaching Center
Turrialba, Costa Rica

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Project Number: 7.095
Grant Number: DPE-5542-G-SS-7034

A.I.D. Grant Project Officer: Dr. Miloslav Rechcigl

Project Duration: July 31, 1987 to August 1, 1990

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EXECUTIVE SUMMARY

The purpose of the research supported by this USAID grant was to apply recent theoretical and technological advances in plant science, in particular the use of DNA molecular markers, to understanding the genetics of the chocolate tree, *Theobroma cacao* . World-wide production of *T. cacao* seeds, used in the manufacture of chocolate and as the source of cocoa butter for the chocolate and cosmetic industries, exceeds one million metric tons and represents a major cash crop for some 40 developing countries located within 20 degrees latitude of the equator. Except in Malaysia where large cacao plantations are maintained, cacao is grown on small farms by the more than one million farmers around the world who are engaged in tending more than one billion cacao trees. The primary long-term objective of our research is to produce high-yielding, disease and insect-resistant, cacao trees through the use of modern molecular genetic technology, for the purpose of providing the small cacao farmer with plant material that will allow him to maintain or increase production levels on much less land than he currently uses, thus freeing fertile ground for growth of food crops.

The work is being done in collaboration with scientists at the Tropical Agriculture Research and Teaching Center (CATIE) in Turrialba, Costa Rica. CATIE was established in 1942 with major assistance from the United States Department of Agriculture mainly through the efforts of Secretary of Agriculture Henry A. Wallace. In recognition of his contribution, the main administration building at CATIE is called the Henry A. Wallace Building. Over the past 50 years, CATIE has trained and awarded Master's Degrees to more than one thousand students from all the Central American and many of the South American countries. Also, CATIE maintains important germplasm collections for cacao, coffee, banana, tropical fruits, pejibaje, and pepper, all of which serve as invaluable research material for students, staff, and visiting scientists from all over the world.

Financial support from this grant has made it possible to establish a laboratory at CATIE equipped to carry out the techniques of modern plant molecular biology, in particular the use of DNA molecular markers, that are the basis for the molecular genetic studies carried out in this work. Furthermore, the laboratory provides a valuable training aid for Latin American students, a new dimension to the CATIE curriculum.

During the tenure of this grant, two students completed thesis research leading to Master of Science degrees, one at Penn State University, and one at CATIE. August, 1988-Marina Mirazon, Penn State "*Theobroma cacao* : Genetic Analysis Using Restriction Fragment Length Polymorphisms". December, 1990-Vicente Febres, CATIE- "Marcadores geneticos en cacao (*Theobroma cacao*,L) asociados con la resistencia a Moniliasis (*Moniliophthora roreri*). In addition, two other students began their thesis work while this grant was active and finished after the grant period ended. October, 1992-Helga Rodriguez, M.S.,CATIE-" Uso de Marcadores Moleculares Para la Construccion de Un Mapa de Lagamiento Genetico en Cacao, *Theobroma cacao*". May, 1993-Joseph Osei, Ph.D., Penn State-"Construction of a Low Density Linkage Map for *Theobroma cacao* L. Using Random Amplified Polymorphic DNA Markers."

RESEARCH OBJECTIVES

Seeds of the cocoa tree, *Theobroma cacao,* are the source of the multi-billion dollar chocolate and cocoa butter industries, and represent an important cash crop for more than 40 developing countries around the world. Dutch traders carried cocoa from South America (center of origin-near the headwaters of the Amazon River) to Africa and Indonesia in the early 19th century, and today by far the largest growing area is West Africa, where in Ghana and Cote d'Ivoire alone, some 2.2 million hectares of land foster growth of more than one billion cocoa trees. The trees are maintained by several hundred thousand small farmers and annually produce over one million metric tons of dried cocoa beans, more than half the world total, and worth over one billion dollars. There is good news and bad news in these figures. The good news is the income of foreign capital; the bad news is the trees are planted on the best land that otherwise could be used to grow needed food crops. A good solution would be to breed for high yielding, disease resistant, cocoa trees so the same, or greater, production might be achieved on less land. The objective of this project, to use cocoa DNA as a marker for plant breeding, was directed towards this goal.

In West Africa, Central, and South America, the islands of the West Indies and South Pacific, as well as many areas of Malaysia and Indonesia, cocoa is grown by private farmers on small plots of land. In almost every case, the farmers are dependent on government and internationally supported research stations to supply them with planting material and with information on agronomic practices, fertilizers, herbicides, pesticides, and how to deal with problems related to viral and fungal diseases. In Ghana these functions are fulfilled by the Cocoa Research Institute of Ghana (CRIG), in Cote d'Ivoire and Togo by the Institute for Research in Coffee and Cocoa (IRCC). In the Americas two of the most important of several institutions serving these functions are the Center for Tropical Agriculture Research and Teaching (CATIE) in Turrialba, Costa Rica, and the Cocoa Research Unit of the University of the West Indies in St. Augustine, Trinidad and Tobago. The latter two institutions maintain the two most complete cocoa germplasm collections in the world. Cocoa breeding programs have been underway in these institutions for more than 50 years with the primary objective of maximizing yield, but progress has been slow, largely because generations times are from five to seven years and programs have lacked continuity. Presently, a mere nine Upper Amazon clones dominate cocoa breeding programs in West Africa and elsewhere, and these apparently were chosen at random from wild cocoa collected by F.J. Pound in 1937 and 1943 (Pound, 1938; Lockwood, 1985).

Utilization of DNA marker breeding technology is highly relevant to tree crops such as *T. cacao*. Long generation times, coupled with the heterozygous nature of the material, dictates that genetic analysis is a complex and time-consuming procedure. These factors have undoubtedly hindered population improvement based on conven-tional plant breeding procedures, particularly for the the many important polygenic traits such as yield, quality, and maturity which are determined by environmental as well as genetic factors. Marker based selection procedures will allow cocoa breeders to apply positive selection for both qualitative and quantitative traits of interest to the cocoa industry at a

much faster rate than has been previously possible using selection on the basis of morphological traits such as disease resistance.

The first alternative to morphological traits for plant breeding selection and for genetic linkage map construction was the use of biochemical markers in the form of isozymes (Tanksley and Rick, 1980). However, there are limitations to isozyme analysis, primarily insufficient numbers of marker loci, and the fact that only expressed genes can be analyzed. The development of restriction fragment length polymorphism
(RFLP) analysis for use in plant genetics removed all such limitations and for the first time made it possible to bring together the skills and knowledge of the classical Mendelian (qualitative) geneticists with the population (quantitative) geneticists (Paterson et al., 1988; Helentjaris and Burr, 1989). More recently, a new DNA polymorphism assay based on amplification of random DNA segments with single polymerase chain reaction (PCR) primers of arbitrary nucleotide sequence, the so-called RAPD technique, has been described (Williams et al., 1990: Welsh and McClelland, 1990). Taken together, isozyme, RFLP, and RAPD analyses are the foundation of marker assisted plant breeding technology (MAPBT) which has already had a major impact on crop science and promises unforeseeable advances in the near future.

Breeding and selection by classical means of a tree crop such as *T. cacao*, with its three to five year generation time, is a most difficult task, but MAPBT should now make it possible to select for genes of interest and, by backcross breeding, introgress such genes into desirable genetic backgrounds. First, and clearly of critical importance, is the time factor. Because selection can be made at the seedling stage, it can be easily calculated that application of MAPBT will reduce the time needed to develop truly elite cocoa germplasm roughly by a factor of two. A second important factor for cocoa breeding is space requirements. In conventional breeding, assuming 3 X 3 M spacing, normally used for cocoa, a 0 05 hectare plot might be required to grow the 50 or so seeds from one pod, whereas with MAPBT, several hundred seedlings can easily be tested in a greenhouse. It should also be pointed out that, in addition to the advantages of time, space, and numbers of testable samples, MAPBT also provides a more accurate selection criteria. The power of MAPBT and the crucial necessity for a *T. cacao* linkage map are perhaps best illustrated by the work of Young and Tanksley (1989) who estimated that by monitoring recombination around genes of interest with linked RFLP markers, an introgressed segment could be obtained in two generations that without RFLP selection would require 100 backcross generations.

METHODS AND RESULTS

I. Restriction Fragment Length Polymorphism (RFLP) Studies

1. *Theobroma cacao*: Genetic Analysis Using Restriction Fragment Length Polymorphisms--a thesis in genetics by Marina L. Mirazon-- in partial fulfillment of the requirements for the degree of Master of Science at the Pennsylvania State University, August, 1988.

This work was the first application of DNA marker technology to *T. cacao*. Detailed protocols for RFLP analysis developed during this work were published in a widely circulated tropical crops journal in the hope that the new technology would be seen as applicable to tropical crops, as well as to the numerous temperate climate vegetable crops where it had been largely developed. One important observation noted in this paper was that a biotin-based non-radioactive labelling system gave results equivalent to classical radioactive labelling methods. This made it clear that the method could be used in cocoa growing countries where research facilities are not equipped for the use of radioactive materials

TURRIALBA 39:519-524, 1989, "*Theobroma cacao* DNA: Protocols for RFLP Analysis, M.L. Mirazon, G. Gora-Maslak, L. McHenry, and P.J. Fritz.

2. Marcadores geneticos en cacao (*Theobroma cacao* L.) asociados con la resistencia a Moniliasis (*Moniliophthora roreri*)-- a thesis presented to the post graduate teaching program by Vicente Jose Febres Rodriguez-- in partial fulfillment of the requirements for the degree of Magister Scientiae at CATIE, November, 1990.

Monilophthora roreri is the causal agent of "watery pod rot", a fungal disease that has devastated cocoa growing areas in Costa Rica and other Central and South American countries. DNA from 11 cocoa clones in the CATIE germplasm collection, 6 displaying resistance to the fungal pathogen *M. roreri*, and 5 having no resistance, was analyzed by RFLP methodology with the purpose of identifying fragments of genomic DNA that were correlated with whole plant resistance to the fungus. The work demonstrated that the gene for a 21 kD cocoa seed protein with trypsin inhibitor activity was highly polymorphic when used as an RFLP probe with these clones. Six groups were found in combination with the restriction endonuclease HaeIII, but there was no correlation with resistance to *Moniliophthora*. The work clearly demonstrated the feasability of conducting RFLP experiments at CATIE, and since the probe was prepared at Penn State, the ability to carry out an intercontinental collaboration was demonstrated.

PHYTOPATHOLOGY 86:608, 1992 (abstract), "Potential Use of RFLP for the Characterization of Cocoa Clones Tolerant to *Moniliophthora roreri*, V. Febres, R. Lastra, V. Villalobos, and P.Fritz.

II. Amplification Fragment Length Polymorphism (AFLP) Studies

1. Uso de Marcaradores Moleculares Para la Construccion de un Mapa de Ligamiento Genetico en Cacao (*Theobroma cacao*)-- a thesis presented to the postgraduate teaching program by Helga Rodriguez in partial fulfillment of the degree Magister Scienciae at CATIE, October, 1992.

In June, 1990, as the period of support from this grant was nearing the end, hand-pollinations were carried out at CATIE to establish a backcross segregating population of cocoa trees for the purpose of using this genetically defined group of trees, along with marker-based technology, to

construct a genetic linkage map for cocoa. The knowledge and insights gained during the course of this grant were the inspiration for establishing the backcross segregating population. The thesis by Helga Rodriguez reported the first use at CATIE of polymerase chain reaction (PCR) methodology, the basis of AFLP studies. The major advantage of AFLP over RFLP analysis is enormous saving of time.

2. Construction of a Low Density Linkage Map for *Theobroma cacao* L. Using Random Amplified Polymorphic DNA Markers--a thesis presented to the Penn State University graduate program in Plant Physiology by Joseph Osei in partial fulfillment of the degree Doctor of Philosophy, May 1993.

The first genetic linkage map for *T. cacao* resulted from work supported in part by this grant. Although the map was done after the grant period ended, the work began while the grant was still in effect and thus benefited from the grant. A finished and working linkage map will be the ultimate accomplishment of the work identified by the title of this grant, "*Theobroma cacao* DNA as a Marker for Plant Breeding". In the work of Joseph Osei, 220 ten-nucleotide long primers of random sequence, but containing at least 50% guanine + cytosine, were used singly in PCR assays to screen for DNA polymorphisms in the parental clones used to produce the backcross segregating population mentioned above. Of the 220 primers tested, 42 markers segregated in such a way to be useful in the Mapmaker (Lander et al., 1987) computer program used for linkage map construction. At a lod score of 3 (meaning the odds are more than 1000 times greater that markers are linked than unlinked) 22 markers were arranged in 6 linkage groups. Cocoa has a haploid number of 10 chromosomes so more markers are needed to complete the map. We have calculated that some 178 markers are needed to have a saturated linkage map but because the cocoa genome is so small (200 megabase pairs, Couch et al, 1993), it may require less.

IMPACT, RELEVANCE, AND TECHNOLOGY TRANSFER

1. How will findings be useful in developing countries?

One of the benefits of the rapidly developing new technology which has revolutionized plant science in the past few years is that the methods are easy to use and relatively inexpensive. For developing countries this means that the technology can be used in these countries without delay. The use of molecular markers for advancement of plant genetics, the main thrust of the work supported by this grant, will have important consequences for development of new and better planting material in the coming years. Although applications to cacao were the subject of the work supported by this grant, it is clear that other crops will also benefit.

2. Impact on individuals, laboratories, departments, and institutions.

The four students who obtained graduate degrees from the work supported by this grant were all from developing countries-Marina Mirazon from Brazil, Vicente Febres from Venezuela, Helga Rodriguez from Colombia, and Joseph Osei from Ghana.

The Biotechnology Laboratory at CATIE was established and equipped with funds made available by this grant. Major items of equipment for this laboratory purchased with funds from this grant include: 1) a -20°C freezer, 2) a crushed-ice making machine, 3) vaccuum oven and vacuum pump, 4) two complete agarose electrophoresis units, including power supplies, 5) Tissue-mizer for preparing plant extracts, 6) a constant-temperature water bath, 7) ultra-violet transilluminator for visualizing DNA in ethidium bromide stained agarose gels, 8) Quick-shooter camera apparatus for photographing gels, 9) various cases of glassware including beakers, flasks, graduated cylinders, etc. 10) various miscellaneous items including spatulas, test tube racks, etc., 11) a Beckman Microfuge centrifuge, 12) four micropipettors 1-10µl; 1-20µl; 1-200 µl; 200-1000 µl.

The Tropical Agriculture Research and Teaching Center near Turrialba, Costa Rica now has a thesis option for it's Master of Science degree program in the area of plant biotechnology as a result of this grant.

3. Will results be used?

The results of the work supported by this grant laid the foundation for the continuing work being pursued at CATIE to use molecular marker technology for construction of a genetic linkage map for *Theobroma cacao.*

4. What difference has the project made?

The project has brought a new dimension to the CATIE curriculum and has made it clear that the new technology can be used to advantage in a developing country with the resources available in Costa Rica. Also, it has demonstrated that collaborations between U.S. Universities and developing country scientists and students can be a productive and rewarding experience.

PROJECT ACTIVITIES/OUTPUTS

1. Meetings

March 12-14, 1990 First International Cocoa Genome Mapping Meeting held at Penn State University. Partial list of attendees:

1. Dr. Claire Lanaud, Head, Tropical Species Genome analysis Laboratory, Centre de Cooperation Internationale en Recherche Agronomique pour le Development (CIRAD), Montpellier, France.
2. Dr. Ramon Lastra, Coordinator of Graduate Programs, Centro Agronomico Tropical de Investigacion y Enseñanza (CATIE), Turrialba, Costa Rica.

3. Dr. Wayne Powell, Head, Department of Molecular and Cellular Biology, Scottish Crop Research Institute, Invergowrie, Scotland.
4. Dr. Raymon Schnell, Germplasm Coordinator, USDA National Clonal Repository, Subtropical Horticulture Research Station, Miami, Florida
5. Peter Greening, Executive Director, International Fund for Agricultural Research, Alexandria, Virginia.

2. Training

In addition to the 4 graduate degrees listed above, additional short-terr training also was accomplished:

a. February 22-March 11, 1988-Dr. Gustavo Enriquez, Cocoa Coordinator, CATIE, visited the ACRI-Penn State Cocoa Molecular Biology Laboratory for instruction in RFLP analysis methodology.
b. February 14-26, 1989-Vicente Febres, graduate student from CATIE visited Penn State for training in RFLP methodology.
c. February 27-March 22, 1989-Teresa Snyder, Penn State Graduate student visited CATIE for instruction in testing for fungal infections of cocoa in connection with the RFLP studies for disease resistance.

3. Publications

a. **TURRIALBA** 39: 519-525 (1989) *Theobroma cacao:* Protocols for RFLP Analysis, Mirazon, M., Gora-Maslak, G., McHenry, L., and Fritz, P.J.
b. **PROC. INTERNATIONAL COCOA RESEARCH CONF.:CHALLENGES IN THE 90'S**, Kuala Lumpur, Malaysia, 1991, Molecular and Biochemical Markers for Genetic Analysis of *Theobroma cacao,* Fritz, P.J., Osei, J.K., Goodin, M.M., and Furtek, D.B.
c. **PROC. BIOTECHNOLOGY FOR TROPICAL CROP IMPROVEMENT IN LATIN AMERICA,** 4th Conf. Int. Plant Biotechnology, San Jose, Costa Rica, 1991. The gene for a 21 kilodalton cocoa seed protein is polymorphic for several cocoa clones. Febres, V.J. Lastra, J.R., Villalobos, V.M., and Fritz, P.J.
d. **PROC. INTERNATIONAL WORKSHOP ON CONSERVATION, CHARACTERIZATION, AND UTILISATION OF COCOA GENETIC RESOURCES IN THE 21ST CENTURY**, Port of Spain, Trinidad, 1992, Characterization: A Requirement for Conservation and Utilization of Cocoa Genetic Resources for the 21st Century, Fritz, P.J., Sain, S.L., Osei, J.K., Snyder, T.E., Dodo, H., Patel, V., Signs, M., Andebrhan, T., and Furtek, D.B.
e. **PHYTOPATHOLOGY** 86:608, 1992 (abstract), Potential use of RFLP for the characterization of cocoa clones tolerant to *Moniliophthora rorei.* Febres, V., Lastra, R., Villalobos, V., and Fritz, P,
f. **IN PREPARATION**, *Theobroma cacao* RFLPs at Trypsin Inhibitor Loci Do Not Correlate with Fungal Resistance, Febres, V., Lastra, R., Villalobos, V.M., Tai, H., Snyder, T., and Fritz, P.J.

PROJECT PRODUCTIVITY

The primary goal of beginning the molecular genetic analysis of *Theobroma cacao* for the purpose of developing disease resistant and higher yielding plants was accomplished. The further goal of establishing a plant molecular laboratory in a developing country and training students and staff in molecular techniques was also accomplished.

FUTURE WORK

The laboratory established at CATIE is currently actively engaged in pursuing the molecular genetic analysis of cocoa under a new USAID Grant.

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