Agricultural Biotechnology for Sustainable Productivity Project

Report to the Technical Advisory Group

April 1997

ABSP

"Agricultural Biotechnology for a Better World"

USAID

Acknowledgements

The reports in this document represent over five years of dedication by the participants of the U.S. Agency for International Development (USAID) Agricultural Biotechnology for Sustainable Productivity (ABSP) Project toward fulfilling the project mission. ABSP is an integrated and complex project which encompasses research in genetic engineering and micropropagation, institutional policy issues of biosafety and intellectual property rights, training in research and associated issues of technology transfer, and networking globally to access and provide shared information.

The research, management and networking team members at developing country and U.S. institutions have jointly produced this document. Thanks to all the co-investigators for their submissions. Members of the network and management teams are given special appreciation for their contributions, with special thanks to Ms. Linda Skiba, ABSP Management Secretary, for her leadership in coordinating, collating, and publishing the document.

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May 13, 1997

Dr. Jeff Hill
USAID Washington
AFR/SD/PSGE
1111 19th Street, Suite 210
Arlington, VA 22209

Dear Jeff:

Enclosed please find five copies of the Report to the Technical Advisory Group which substitutes for the Quarterly/Annual Progress Report for the ABSP project per Dr. Judith Chambers, USAID. This report covers the quarterly/annual period January 1, 1997 - March 31, 1997.

This report includes information on the technical, managerial and financial aspects of the ABSP project.

Please feel free to contact me if you require any additional information or clarification.

Best regards,

Catherine L. Ives

Catherine L. Ives
Co-Managing Director
ABSP Project

CLI/lw

Attachment

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Introductory Remarks

Donald Isleib

Chairperson, ABSP Internal Board of Directors

This report summarizes accomplishments of the Agricultural Biotechnology for Sustainable Productivity (ABSP) Project through February 1997. The results reported reflect the combined efforts of many people who have contributed their skills and efforts to this project. We are proud of their achievements, which have met or exceeded the goals identified in the project plan.

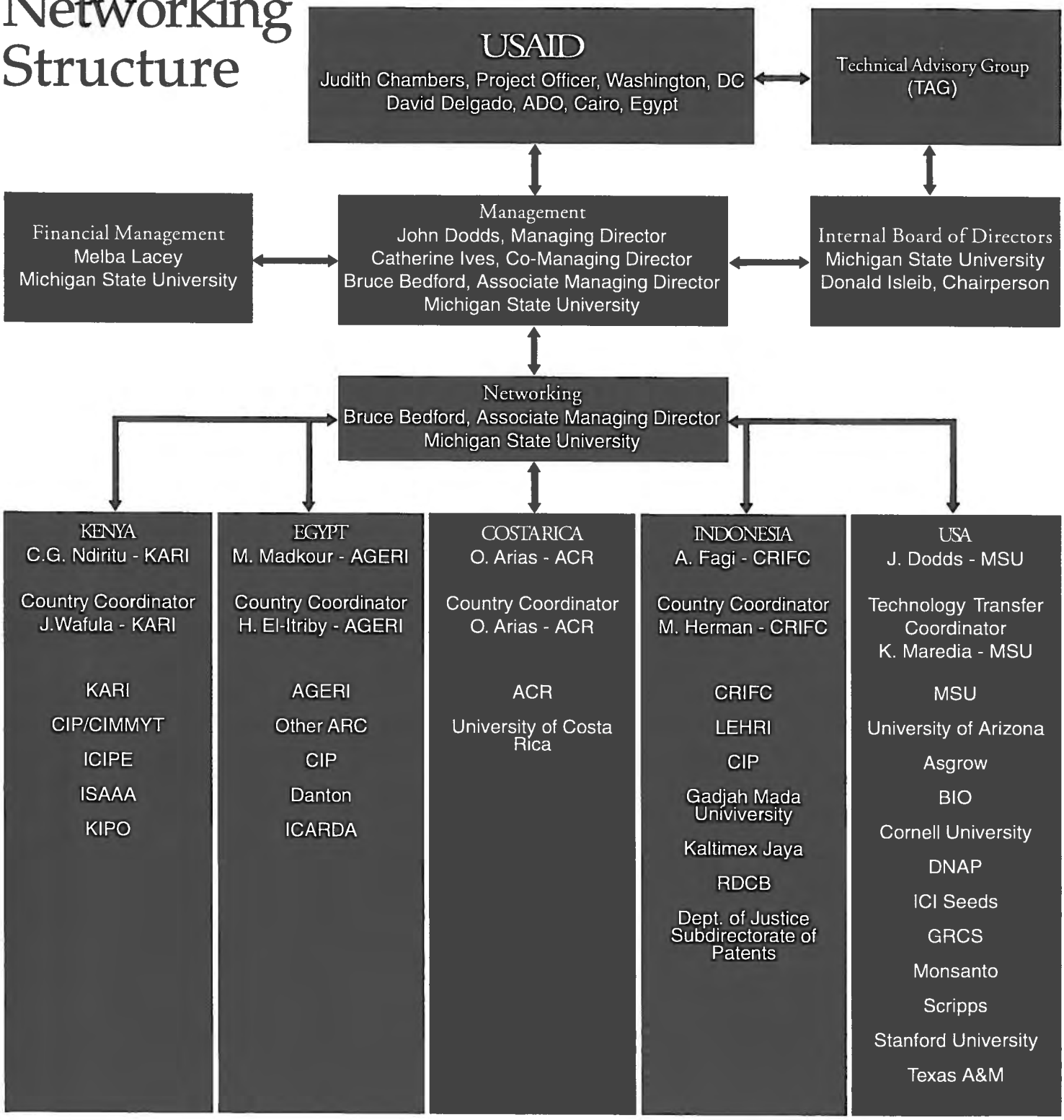
We are especially proud of accomplishments in the area of technology transfer - that is, exchange of personnel, transfer of knowledge and research tools, and introduction of transformed cultivars to partner developing countries - based on institutional linkages which have been created and nurtured among widely-diverse organizations and their scientists and administrators.

The project has been successful in building a team of competent and motivated people, both in the U.S. and in partner developing countries. This human resource development effort has included researchers, policy analysts, and administrators.

As ABSP has progressed from its initial focus on transformation of targeted species to the current focus on transfer of these species to field testing and commercialization, important elements addressing provisions for protection of intellectual property rights and biosafety have increased in importance.

Continued dependence on participation by both U.S. and host-country scientists, policy makers, and administrators will be crucial to the continued success of the ABSP Project. We expect the new project manager and her team to sustain the progress made to date, and we look forward eagerly to future achievements.

ABSP Research, Management and Networking Structure



Summary of Project Outputs as Defined by USAID

Accomplishments of the ABSP Project Through 1996

Catherine Ives
Michigan State University

The Agricultural Biotechnology for Sustainable Productivity (ABSP) Project is on track to meet the initial project outputs as defined in the original project paper developed by the U.S. Agency for International Development, Office of Agriculture and Food Security. A summation of accomplishments, set against the original project outputs and indicators, is given below.

Priority Research and Technology Transfer

Output: *Develop Multi-Disciplinary Applications of Biotechnology for Tropical Crop Improvement*

Indicator: *Three Recombinant and/or Tissue Culture Technologies Developed for Tropical Crops*

The ABSP project has developed micropropagation methods for banana and pineapple. In addition, the ABSP project has developed transgenic melons for resistance to zucchini yellow mosaic virus (ZYMV), transgenic maize with putative resistance to Asian stem borer (ASM), transgenic tomatoes with resistance to geminivirus, and transgenic potatoes with putative resistance to Potato Tuber Moth. In addition, ABSP has joined in a collaboration between Monsanto and the Kenyan Agricultural Research Institute (KARI) to develop sweetpotato with resistance to sweetpotato feathery mottle virus (SPFMV). All these products have been developed in collaboration with our host country partners in a multi-disciplinary manner, with the training of breeders, molecular biologists, entomologists, and tissue culture specialists (see below).

Output: *Develop Publications and/or Patents as Documentation of Technical Accomplishments*

Indicators: *Three Patents; Six Publications*

The ABSP project has supported the publication of 41 papers. In addition, two patents have been filed for research under ABSP for methods for the *in vitro* sexual reproduction of corn plants and a method for asexual *in vitro* propagation of corn plants.

Output: *Small-Scale Field Tests of Biotechnology-Derived Plants Conducted in the U.S. and LDCs to Establish Agronomic Acceptability of Germplasm*

Indicators: *Nine Field Tests of Biotechnology-Derived Plants Conducted in the U.S. and LDCs*

The ABSP project has sponsored confined field tests for transgenic potato, transgenic melons and transgenic maize in the U.S. Twenty-nine transgenic potato lines from 7 different potato cultivars were in various years in Michigan, California, and Egypt (see potato research results). Additionally, several trials of transgenic cucurbits have been conducted in Michigan and

New York (see cucurbit research section). Twelve maize lines were initially selected for transgenic research, which were narrowed down to three lines for continued research in the Midwest region of the U.S. (see maize research section).

One of the major difficulties encountered in the project has been obtaining clearance from USAID for transfer of these materials to developing countries for continued field tests. In order to rectify this problem, ABSP hired as a consultant, Dr. Patricia Traynor, who is an expert in biosafety, to assist us in obtaining these approvals and developing appropriate field testing protocols for our developing country partners.

In January 1997, the ABSP project received permission to transfer transgenic potatoes to Egypt. Experiments are underway to assess the resistance of these varieties to potato tuber moth.

***Output**: Registration and Distribution of Improved Germplasm Proven to be Field-Tolerant for Adverse Pests, Pathogens, and Environments*

***Indicators**: Twelve Improved Genotypes More Tolerant of Adverse Environments, Pests, and Pathogens Distributed*

ABSP has produced more than 12 improved genotypes which are likely to be more tolerant of adverse environments, pests, and pathogens. The final analysis of this, however, awaits testing in the fields of our host country partners. The following genotypes have been developed:

- **Cucurbits (melons):**

  Hale's Best Jumbo - CP

  Topmark - CP

  Ananas El Dokki - CP

  Shahd El Dokki - CP

- **Cucurbits (squash):**

  C. papo - CP of SqMV

All these melon and squash cultivars have been engineered for resistance to zucchini yellow mosaic virus (ZYMV) and squash mosiac virus (SqMV), respectively, by using the ZYMV coat protein.

- **Potatoes:**

  FL1607 - Cry1A(c)

  Lemhi Russet - Cry1A(c) - Codon Modified

  Atlantic - CryV - Codon Modified

  Russet Burbank - Cry1A(c) - Codon Modified

  L235-4 - Cry1A(c)

These potato lines contain either the CryIA(c) wild type gene, the CryIA(c) codon modified gene, or the CryV codon modified gene. The Bt genes should confer resistance against potato tuber moth; final analysis awaits field testing and greenhouse testing in host countries.

- **Maize:**

  A188xBE73 - CryV

  A188xBE73 - CryIA(c)

  NP19 - CryIA(c)

  NP19 - CryV

These two maize lines have been transformed with both the CryIA(c) and CryV genes *of B. thurengensis* to produce maize resistant to Asian corn borer. Final efficacy and agronomic characteristics await field testing in Indonesia.

- **Tomato:**

  UC82B

The above genotype has been studied and engineered for resistance to geminiviruses using the coat protein, the pre-coat protein, the replicase gene or the AV4 gene of Egyptian geminiviruses. More than 267 transgenic tomato lines have been established (see tomato research section).

- **Sweetpotato:**

  Jewel - cp SPFMV

  CPT-560 - cp SPFMV

This project has been primarily supported by Monsanto Company through a contract with USAID. Participation of KARI personnel has been supported by MIAC and ABSP. ABSP hopes to assist in transferring this technology to Kenya for adaptation and testing. These lines have been transformed with the Sweetpotato Feather Mottle Virus (SPFMV) coat protein and are expressing the transgene. The CPT-560 is an African variety and Jewel is an American variety. They await testing in host country before confirmation of resistance to Kenyan strains of SPFMV can be demonstrated.

Output: *Marketing Analysis, Feasibility Study, Biosafety Regulatory Status in LDCs; Economic Impact Analysis*

Indicators: *One Analysis, One Feasibility Study, One Biosafety Study; Conduct Two Economic Impact Analysis for Project Outputs*

An economic impact analysis for the micropropagation technologies developed with our private sector partners in Costa Rica and Indonesia has been conducted. We have invested considerable resources in assisting our partners in developing biosafety regulatory policies and procedures so that they will be able to effectively, efficiently, and safely use the products developed from the ABSP project and elsewhere. In addition, Dr. Traynor has developed a proposal for the transfer and handling of transgenic material to Egypt. This proposal will serve as a model for future transfers of transgenic material. Marketing analyses have not been conducted to date.

Innovative Activities

Output: *Establish Commercial Links for Appropriate Technologies Developed Between U.S. Companies and Universities and LDC Institutions*

Indicators: *Two Commercial and Managerial Links Developed Among U.S. Companies and LDC Institutions, and Three Industry-Based Management Seminars*

The ABSP project has been very successful in establishing commercial linkages for different technologies between U.S. companies and universities and LDC institutions. The project has supported a commercial linkage between DNA Plant Technology (Oakland, California) and Agribiotecnologia de Costa Rica (ACR), and between DNAP and Fitotek Unggul (Jakarta, Indonesia). In addition, we have supported a partnership between ICI Seeds, now Garst Seeds (Slater, Iowa) and the Central Research Institute for Crops (Bogor, Indonesia). We have also supported, in part, a relationship between the Kenyan Agricultural Research Institute (KARI) and Monsanto Company (St. Louis, Missouri), although the initial USAID collaboration was through MIAC. The ABSP project has also sponsored an informal linkage between Asgrow Company (Kalamazoo, Michigan) and the Agricultural Genetic Engineering Research Institute (Giza, Egypt).

We have conducted only one industry-based management seminar in April 1993. The other planned seminars were postponed due to budgetary constraints. We hope to conduct these seminars during a proposed Phase II of ABSP.

***Output**: Increased Awareness of LDC Officials Through Internships of IPR and in Development of Regulatory Policy Through Positions in the U.S. Regulatory Agencies*

***Indicators**: Six Internships Provided for (a) Protection of IPR in U.S. Legal Firms (4); and (b) Scientifically-Based Biosafety Regulatory Policy in U.S. Regulatory Agencies (4)*

We have exceeded this goal. During the course of the project, we have placed more emphasis on the policy areas since it quickly became apparent that the lack of appropriate biosafety regulations and intellectual property systems were serious deterrents to transferring these technologies to developing country partners.

In May 1993, ABSP sponsored a biosafety internship program with seven participating scientists from Egypt, Kenya and Indonesia. The participants reviewed current biosafety regulations and guidelines, became familiar with various biosafety issues related to the safe handling of transgenic germplasm in the laboratory, greenhouse and field, developed a draft set of guidelines and regulations for their own countries, and learned about perceived risks and benefits of a biosafety regulatory framework.

In April 1993, ABSP, in cooperation with Stanford Law School, sponsored a four-week internship program which covered both plant breeders' rights and regular patents as they apply to genetic engineering of plants. The program included substantial discussion of patent licensing, research agreements, and explored policy issues of patent laws for developing

countries. The project sponsored two participants from Indonesia, two from Kenya and three from Egypt.

Since this initial focus on biosafety and IPR, the ABSP project has sponsored numerous other workshops and internship programs on these topics. In the biosafety area, ABSP sponsored a Latin American/Caribbean region and a Middle East region biosafety workshops in 1993 and 1994, respectively. ABSP participated in an Africa Region Biosafety meeting in Harare, Zimbabwe, which was organized by the Netherlands Development Assistance Agency.

In May 1996, ABSP participated in a biosafety capacity building workshop in Sweden at the Stockholm Environmental Institute.

In addition, ABSP has sponsored participation in biosafety workshops organized by ISAAA in Indonesia and Kenya.

In August 1996, ABSP, in cooperation with the Information System for Biotechnology at Virginia Polytechnic State University, organized a two-week internship program in biosafety. This program was designed to give participants a thorough grounding in all aspects of biosafety for environmental release of genetically engineered organisms. ABSP sponsored the participation of 11 scientists from Egypt, Costa Rica, Kenya, and Indonesia.

ABSP has also increased its emphasis on intellectual property education and awareness. In January 1994, ABSP organized an in-country IPR workshop in Cairo, Egypt. The workshop addressed patent issues related to materials used in and generated by biotechnology projects. The workshop involved scientists, legal professionals and government officials primarily from Egypt. A second IPR workshop was held in Washington, DC in July, 1994. Invited participants were from Brazil, Egypt, Kenya, Indonesia, Costa Rica, Morocco and Thailand as well as a number of institutions and agencies such as The World Bank and USAID.

Output: *Co-Ordination with Other USAID Projects*

Indicators: *Co-Sponsor One International Conference with One USAID and/or CRSP, One CRSP and One Natural Resource Representative Appointed to the TAG, One USAID Mission Requested Technical/ Institutional TDY Support*

The ABSP project has jointly sponsored the Latin American/Caribbean regional biosafety workshop in Jamaica with the Bean/Cowpea CRSP (May, 1993).

The ABSP project selected Dr. Ronald Carroll of the University of Georgia to be a member of the Technical Advisory Group (TAG). Dr. Carroll is a member of the Sustainable Agriculture and Natural Resource (SANREM) CRSP and is a recognized expert in ecology. In addition, ABSP has sought the expertise of the International Agricultural Research Centers (IARCs) by

selecting Dr. Mujeeb Kazi from CIMMYT and Dr. Gurdev Khush from IRRI to be members of the TAG.

The ABSP project has been very successful in tailoring its goals and expertise to USAID mission needs. The ABSP project has enjoyed continued support from USAID/Jakarta (approximately $1.6 million) and from USAID/Cairo (approximately $4.4 million in a separate grant). In addition, ABSP has received smaller "buy-ins" from USAID/Rabat ($200,000) and USAID/Africa Bureau ($300,000).

***Output**: Strengthen Ties Among LDC Institutions and Private Institutions, and Commercial Biotechnology Trade Association*

***Indicator**: Co-Sponsorship of Seven Memberships in a Biotechnology Trade Association*

Our developing country partners have received full memberships in the Biotechnology Industry Organization (BIO). This includes Scripps Research Institute, KARI, CRIFC, Fitotek Unggul, ACR, and AGERI.

***Output**: Provide Legal Expertise to Project's Institutions*

***Indicators**: Two USAID Provided Technical Support, IPR, Biosafety Internships or Consulting, Network Activities; Write and File Three Patent Applications from Project Research and/or Licenses*

The ABSP project has placed considerable resources in the area of legal consultation and training. (See the above section on increased IPR and biosafety awareness.) In the area of legal expertise, ABSP has, when needed, procured the services of MSU's Technology Transfer Office and MSU's patent attorney. In addition, Professor John Barton, acting as an IPR consultant to ABSP, has actively assisted developing countries in designing and implementing IPR legislation.

To further assist developing countries in developing and managing their intellectual property, ABSP and the MSU Office of Intellectual Property organized a two-week internship program in IPR and technology transfer in February 1996. Eleven participants from eight different countries attended. The program provided "hands on" experience to participants in the day-to-day handling of intellectual properties in university and private sector settings.

The project organized an in-country IPR workshop in Indonesia in 1996. Over fifty scientists, legal personnel, administrators and senior government officials participated in this workshop. The goal of the workshop was to discuss the development of new Plant Variety Protection laws in Indonesia.

Finally, USAID/Rabat requested project assistance in sponsoring a workshop in Morocco on Plant Variety Protection (PVP). This

workshop, held in March 1997, addressed regulations and implementation issues surrounding Morocco's newly passed PVP law.

The project has supported the filing of two patents (U.S. 5,281,529 and U.S. 5,320,961) as mentioned above.

Output: *Environmental Analysis, Field Testing and Germplasm Distribution*

Indicator: *Prepare Three Reports for Environmental and Field Testing Protocols*

The ABSP project has prepared a report for USAID's Biosafety Committee for the field testing of transgenic potatoes, transgenic melons, and transgenic tomatoes in Egypt. A similar report will be prepared for field testing in Indonesia.

Extensive environmental analyses has not been conducted to date due to: (1) budgetary constraints; and (2) voluminous analysis prepared by USDA and others regarding the use and deployment of coat protein and Bt technology. However, if funds are available, the ABSP project will conduct an environmental analysis in one or more host countries, focusing on potential benefits of the technology with regard to pesticide usage. ABSP did, however, partially sponsor research work by Rebecca Grumet's laboratory exploring transgenic pollen flow and associated risk.

Other Innovative Activities

The ABSP project assisted in building a biosafety containment greenhouse in Egypt. ABSP managed the contract between USAID/Cairo and the University of Arizona. The greenhouse has been completed and certified by the USDA (August 1995).

Network Activities

Output: *Provide Support to Three LDC Tissue Culture Facilities to Enhance Their Technical Capabilities, Increase Efficiency in Biotechnology, Integrate Research with Conventional Plant Breeding, and Serve as Facilitators*

The ABSP project supported commercial tissue culture facilities in Indonesia and Costa Rica (Fitotek Unggul and ACR, respectively). We have also provided technical assistance to CRIFC, KARI, Kenyatta University (Kenya) and AGERI to develop the trained personnel needed to carry out improved tissue culture technology. The ABSP project also provides limited "core" funds to our developing country partner institutions for needed equipment and supplies to continue to develop these technologies independently.

Output: *Convene International and Regional or Country-Specific Conferences*

Indicators: *One International Conference to Include Workshop on Research Management, Three Regional or Country-Specific Conferences*

The ABSP project has exceeded this goal. The project has held the following workshops:

- Latin America/Caribbean Regional Biosafety Workshop, May 1993, Jamaica.
- Biosafety/IPR/Project Review Workshops, January 1994, Egypt.
- IPR Workshop, July 1994, Washington, DC.
- Mediterranean Regional Genetic Diversity Workshop, June 1994, Egypt.
- ABSP/Indonesia Technology Transfer Workshop, April 1995, Indonesia.
- Indonesia IPR Workshop, March 1996, Indonesia.

We have not conducted a workshop on research management, but in January 1997 we began developing a Memorandum of Understanding with the Intermediary Biotechnology Service (IBS) at ISNAR. IBS has developed a workshop on research management and, if appropriate, ABSP will work with IBS to provide this training to project personnel in developing countries.

***Output**: Follow-On Support Provided to Trained Scientists and Their Institutions*

***Indicators**: Three Scientists Provided Follow-Up Support*

The ABSP project was designed to provide follow-on support to the scientists that have been trained under the project through their host country institutions. We have already provided, or will provide shortly, funds for CRIFC/Indonesia, KARI/Kenya, AGERI/Egypt, and ACR/Cost Rica. There have been some difficulties in developing the appropriate contractual arrangements with these host country institutions due to MSU and USAID's requirements to show "evidence of responsibility," i.e. for the host country institutions to demonstrate that they have sufficient management and accounting systems to withstand a potential audit. For developing countries, documentation deemed appropriate by USAID in English can be difficult. This has slowed establishment of these subcontracts and has delayed in-country research efforts. However, all collaborating institutions now have subcontracts in place, and in-country research has begun.

In addition, the project has supported a small grants project in Indonesia. Four scientists from different institutions have been supported to study sweetpotato regeneration, micropropagation of ginger, detection of pod rot disease in cacao, and the development of virus-free citrus seedlings through protoplast fusion.

***Output**: Provide Biosafety Guidance to LDCs*

***Indicator**: Three Biosafety Guidances Provided*

Biosafety guidance has been provided to our host country collaborators on three levels:

- one-to-one consultations,
- in-country workshops or sponsorship to attend other workshops, and
- more in-depth internship training.

Output: *Provide Support to Developing Publications from Research Conducted at Three Laboratories*

Indicator: *Three Publications Developed*

Since in-country research has only recently begun, no publications from developing countries have yet been developed.

Output: *Provide Support to CGIAR Centers for Regional Coordination*

Indicator: *Three CGIAR Centers Identified and Supported for Regional Network Activities*

The ABSP project, due to budget constraints, was forced to re-prioritize its networking activities. Unfortunately, support to CGIAR centers could not be maintained under these budget pressures. A decision was made to focus directly on national program needs. ABSP hopes to re-visit support to CG centers as budget and time allows. However, ABSP's Technical Advisory Group does include two members from the International Centers (Dr. Gurdev Khush at IRRI and Dr. Mujeeb Kazi at CIMMYT). In addition, we have sought the collaboration with centers regarding the testing of the transgenic material that we have produced at in-country locations.

Training

Output: *Post-Doctoral and Graduate Degree Level Training in Molecular Biology and Biotechnology in Either Public or Private Sector Research Facilities*

Indicators: *Six Post-Doctoral Candidates Trained, and Two Graduate Degree Level Scientists Trained*

The ABSP project made a decision not to focus on degree training in the project due to insecurity of funding and in order to allow the project maximum flexibility in meeting USAID/Washington, USAID/Mission, and host country partner demands. However, ABSP did support one graduate student, Dr. Duncan Kirubi, who received his Ph.D. from Texas A&M University in September 1996 and has returned to Kenya to continue his work on maize transformation. Additionally, the ABSP project has trained nine post-doctoral candidates for time periods ranging from six months to two years.

The ABSP project has also emphasized short-term training. To date, we have supported over 58 scientists, administrators, and policymakers in various aspects of biotechnology policy (biosafety and IPR), research and networking through participation in short-courses, short-term training, workshops, and internships.

Output: *Research to Appraise Use of Biotechnology in the U.S. as Related to Applications in LDCs*

Indicators: *Three U.S. Biotechnologies Appraised for Applications in LDCs*

The three US biotechnologies which were appraised for application in LDCs were:

- bioreactor micropropagation technology,

- coat protein technology to develop transgenic crops resistant to virus infection, and
- use of Bt genes for resistance to specific pests of corn and potato.

These technologies have all been employed in various crops and are in the process of being transferred to developing countries.

ABSP

Laboratory Research Progress

Development of Asian Corn Borer Resistance in Tropical Maize - U.S.

Jan Tippett
Pam Robeff
Diane Gingrich-Feil
Brad Coates
ICI Seeds, Inc.

Former Researchers
Martin Wilson
Bahagiawati Amirhusin
Kim Hagemann
Bruce Held
Firdaus Kasim
Edy Listanto
Saptowo Pardal

Introduction

Bacillus thuringiensis (BT) has been used extensively for the control of insect pests both as a formulated biocontrol agent, and more recently *via* plant transformation with specific endotoxin genes. In the U.S. *Ostrinia nubilalis* (European corn borer; ECB) is a main pest of maize and its control with BT proteins has been well documented (Shnepf et al., 1985; Ge et al., 1989). A close relative, *Ostrinia furnacalis* (Asian stem borer; ASB) is a pest in Indonesia, where its lifecycle allows it to threaten maize virtually year round. The aim of this project is to produce resistant germplasm using the proprietary Zeneca BT gene cryV, which was known to be active against ECB and later shown to be similarly active against ASB. The project is nearing completion having achieved two of the three objectives outlined below. There are biosafety and intellectual property (IP) constraints which have so far prevented the transfer of transgenic plant material to Indonesia.

Project Objectives

1. Production of tropical corn with resistance to *Ostrinia furnacalis* (Asian stem borer, ASB) via transformation with endotoxin genes derived from *Bacillus thuringiensis.*
2. Transfer of enabling technologies to Indonesian scientists *via* training in the U.S.
3. Commercialization of insect resistant germplasm generated from the project.

Tropical Corn

Research Progress and Achievements

The technical aspects of the production of tropical corn with resistant to *Ostrinia furnacalis* (Asian stem borer, ASB) via transformation with endotoxin genes derived from *Bacillus thuringiensis* can be broadly divided into the following five areas:

1. Selection of germplasm for transformation.
2. Selection of resistance genes and construct components.
3. Transformation.
4. Analysis of transformants.
5. Introgression into breeding program.

Selection of Tropical Germplasm

Early research involved the selection of tropical germplasm suitable for transformation and a tissue culture study of a variety of maize lines was conducted. Bahagiawati Amirhusin was our first visiting scientist and she conducted the initial research using 12 inbred lines from both Zeneca's portfolio and other tropically adapted material. Unfortunately, quarantine restrictions prevented the use of Indonesian germplasm at that time and it was agreed that the BT transgene would be introgressed into local (Indonesian) material via breeding programs at a later stage.

Primary objectives were to evaluate embryonic initiation, embryogenic-proliferative capacity and regenerative capacity. There were problems with some lines not reaching maturity in the U.S. Midwest region. Lines selected for further study, based on the above criteria, were PN2119, YB2113 and LM2112. Those eliminated were HN2193, PN2116, WM2154, EV2150, LM2111, UM2160, UM2141, MN2151, UM2198.

Selection of BT Genes and Construct Components

The main considerations were activity against the target species *O.furnacalis*, access to BT genes, codon optimization of BT genes, promotors and selection systems. Activity studies against the proxy *O.nubilalis* and the target *O.furnacalis* performed using CryV and CryIA(c) showed that both gene products were effective against both species. The overall intent was to use the cryV gene for the end product; however, while the codon optimization was in progress we were able to use a construct containing CryIA(c) donated by Dr. Pam Green (Michigan State University) for initial research and proof of concept. The cryV gene was optimized under contract in the following way:

	Wild Type	Optimized
AA Homology	-	100%
DNA Homology	-	69%
GC Content	37%	62%
Poly A Sites	24	2
ATTTA	7	6

A review of promotors was conducted based on tissue specific and constitutive expression. Among promotors tested for their suitability were CAB (chlorophyll ab binding), MR7 (maize root), CaMV 35S and maize polyubiquitin. At the time of evaluation no patents had issued in the U.S. for the CaMV 35S and maize polyubiquitin promotors; however, in ~October 1994 Monsanto Company was granted a patent for the CaMV35S promotor and we abandoned its use. The research construct used after that time was pAID5pUB (cryV gene driven by the maize polyubiquitin promotor).

Further changes were made to the final constructs so that there were no antibiotic genes present in accordance with company policy. The final constructs used are designated pIGPDCV and pIGPDC5 (polyubiquitin-cryV gene cassette in two orientations with respect to the plant selectable marker).

Transformation

1. Stable transformation of A/B cell suspensions and PN2119 zygotic embryos via bombardment.

All early transformation work was conducted using the biolistic gun (research only license) and transgenic plants generated were from both A188xB73 and PN2119 germplasm. A total of 60 gun-derived cryIA(c) plants and 40 cryV plants in A188 x B73 background, and 163 gun-derived cryIA(c) plants and 3 cryV plants in PN2119 germplasm, were generated.

However, as the project progressed, it was decided to change over to the Zeneca proprietary 'Whiskers' transformation system in order to overcome IP issues relating to the use of the biolostic gun, which could only be used to generate research, noncommercial material. The system, although less efficient than the gun, has the distinct advantage of circumventing the DuPont gun patent. Whisker clones can only be generated using AxB cell suspensions and conventional breeding will be used to introgress the insect resistance trait into appropriate germplasm.

2. Stable transformation of A/B suspension cells via whiskers technology.

Transformation with whiskers using the constructs pIGPDCV and pIGPDC5 ended in August 1996. This completed the front end effort to generate enough expressing events from which to select plants for progression into a breeding program. It is anticipated that associated downstream tissue culture and regeneration will continue through mid-1997. A summary of events generated follows:

Plasmid	Bt (+) Events	Bioassay (+) Events at Plant stage
pIGPDCV	370	147
pIGPDC5	225	90
Total	595	237

The number of plants regenerated per event varies from 5-15 depending upon clone health and corn borer activity. Activity detected by detached leaf feeding assays in primary transgenics is only an initial screen. The numbers above reflect 1^0 bioassay positive events and plants derived from these events may produce a range of seed counts from 0 - > 300. Fertility and subsequent seed production significantly reduce the number which go forward for field efficacy testing, generally only plants producing >/= 50 seeds are progressed.

Plant Analysis

1. Field efficacy testing.

Plants from five separate events were tested in the field in 1996 for efficacy against 1st and 2nd generation European corn borer. Several plants from each event were planted and evaluated at the whorl stage for 1st generation corn borer and those which demonstrated little or no feeding damage were re-infested at anthesis. Final scoring was performed after harvest by splitting whole stalks and measuring the actual length of the tunnel caused by corn borer larval feeding.

Mendelian segregation is also important at this stage, so that the future inheritance patterns of any successful candidate entering a breeding program can be more readily predicted. All primary transgenics are crossed as males and females, as fertility allows, to an inbred line

related to the B parent and therefore at this screening level segregation should be 1:1. This was observed in only one plant from one event. Low fertility levels and subsequent low seed set can be problematic when trying to determine actual segregation ratios.

There are many more events which are being prepared for the 1997 field efficacy tests and emphasis has been on improving seed set so that segregation ratios can be more accurately determined.

2. Laboratory analysis.

Events from the 1996 field trials are now being looked at for copy number, rearrangements and protein expression levels. Southern analysis has shown copy number to vary between 1 and 5 for events tested to date, and plants derived from the same event appear to have a consistent number of copies.

Introgression into Breeding Program

Further discussion is required with respect to incorporating any potentially useful event(s) into a breeding program. Currently there are still biosafety constraints preventing the transfer of transgenic maize into Indonesia, but regulations to overcome this are being developed. Additional issues include germplasm for transfer, IP surrounding the construct and maize as a protected crop in Indonesia.

Transfer of Enabling Technologies

The first component of technology transfer to Indonesian scientists via training in the U.S. involved training of visiting scientists in transformation and related technologies. The main training areas accomplished were:

- corn transformation,
- cell culture of tropical germplasm,
- insect bioassays - diet, transient callus, whole plant,
- molecular characterization - PCR, ELISA, and
- field evaluation and artificial infestation.

Commercialization of Insect Resistant Germplasm

There have been discussions regarding the best way forward to commercialization of insect resistance germplasm generated from the project with this objective involving a potential commercial collaboration with Kaltimex Jaya, Indonesia. It was determined that at this time there is no market for insect resistance corn hybrids in Indonesia because most corn is of the open-pollinated varieties. However, marketing in the Philippines is a possibility (Kaltimex Jaya).

Dr. Achmad Fagi, Director of the Central Research Institute for Food Crops (CRIFC), is also exploring possibilities for hybrid maize production in Indonesia and this could also provide a commercial avenue.

Constraints/Problems

There have been no significant technical difficulties that have not been overcome or circumvented and most constraints have been in the regulatory and intellectual property arenas. Most of these issues have been mentioned above, but in summary include: crop protection and biosafety regulations in Indonesia, and intellectual property issues with respect to construct components and germplasm.

ICI/Garst has become part of a joint venture named Advanta (jointly owned by Zeneca Inc., U.K. and Suker Unie, Netherlands) and all permissions and notifications relating to CryV and other Zeneca owned entities must now be made through Zeneca U.K. This should not cause problems, but paperwork may require extra time to process.

Continued Needs

This year's field testing will be conducted by Garst's entomology team but there will be a need to hire seasonal help for the sampling and preliminary analysis of the plant/event combinations under evaluation. Selection of suitable events for introgression will continue after the completion of the field trial and then go forward into an appropriate breeding program. It is probable that three-way crosses will need to be made before transferring the transgenics to CRIFC and further discussions are required to determine how these will be made. An update on the current status of progress being made toward hybrid corn production in Indonesia will assist both transfer for continued research and any potential commercialization.

Maize Transformation for Development of Stem Borer Resistance - Egypt

Magdy Madkour
Hanaiya El-Itriby
Ebtissam Hussein
Mamdouh Idriss
Yehia Osman
Agricultural Genetic Engineering Research Institute

Project Objectives

1. Transfer technologies from U.S. counterpart to establish a system(s) for regeneration and transformation of Egyptian maize lines.
2. Production of genetically engineered maize resistant to stem borers specifically *Sesamia cretica* (pink corn borer), via transformation with an insect resistance endotoxin Bt gene.
3. Develop laboratory rearing for the lepidopteran pink borer, *Sesamia cretica*.
4. Establish methods for laboratory bioassays and field testing.

Research Achievements

Technologies for regeneration and transformation of maize were introduced to Agricultural Genetic Engineering Research Institute (AGERI) under the ABSP/AGERI project. Two different methods are used for the transformation of the Egyptian maize lines, i.e. immature embryo culture and multiple shoot meristems.

Immature Embryo Culture

1. Immature embryos cultured on MS or N6 media produced mainly type I calli in the ten maize inbred lines surveyed. The regeneration frequency varied among the different genotypes. Therefore, in an attempt to improve the type of callus for regenerability a new callus induction medium was tested. This medium produced embryogenic calli for some of the tested lines especially line 8 and 9, (Fig. 1).

Fig. 1. Embryogenic callus in maize inbred line 9.

2. a. To improve the transformation efficiency, an experiment was conducted to study the effect of exposing embryos to different osmotic treatments 4hrs prior to their bombardment and 16hrs after bombardment. Also, other conditions were taken into consideration (i.e. pressure and number of shots). Results showed that pretreatment with 0.2 M sorbitol+0.2M manitol was more effective than the use of 6% sucrose. This osmotic treatment along with the

use of 1300 psi rupture disc and two shots gave the best results, (Fig. 2).

b. A kill curve experiment was initiated using different concentrations of ppt in the selection medium. The results revealed that 0.5 mg/L ppt was the most suitable concentration for the selection of herbicide resistant calli.

c. Transformation of immature embryos from lines 1, 2, 8 and 9 was performed on a regular basis using plasmids PTW-a and pRAC-15. A total of 12,150 embryos were bombarded and cultured on selection medium containing 0.5 mg/L ppt. Callus pieces were sub-cultured every two weeks for two months before being transferred to the regeneration medium which also contained 0.5 ppt. (Fig. 3).

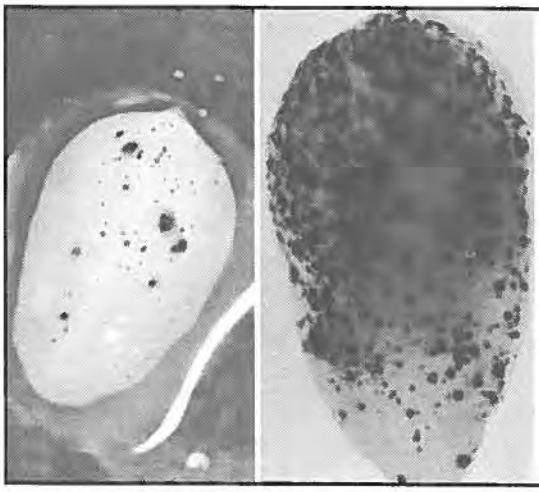


Fig. 2. GUS expression in transformed immature embryo with plasmids pTW-a and pRAC-15 using different osmotic pretreatment (1) control (2) pretreated with 0.2 M sorbitol + 0.2 manitol 4 hrs before bombardment and 16 hrs after bombardment.

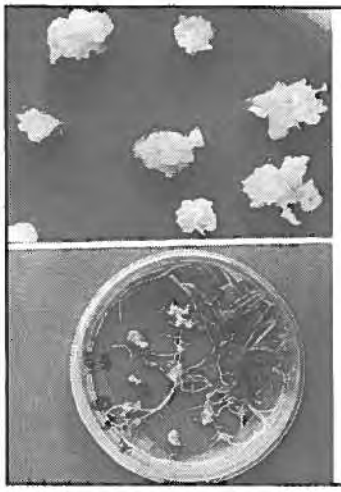


Fig. 3. (1) Putatively transformed calli cultured on selection medium containing 0.5 mg/L ppt. (2) Regeneration of putatively transformed calli on regeneration medium containing 0.5 mg/L ppt.

d. A number of putatively transgenic calli and R0 plantlets have been subjected to different tests including:

- PCR analy-sis of DNA using GUS primers.

- Histo-chemical GUS assay on roots.
- Herbicide application.

Multiple Shoot Meristem System

Multiple shoot meristem system for maize regeneration was used as a non-genotype specific method to overcome the genotype restriction of callus induction and type of callus encountered with the immature embryo culture system (Fig. 4).

Fig. 4 Induction of multiple shoot clumps (line 8).

Nevertheless, variation in the ability and rate of shoot multiplication was observed among the lines surveyed. A lower concentration of phytagel (3 g/L) in the shoot multiplication medium showed a great improvement in the multiplication frequency. Currently, subculturing of multiplied shoot clumps is routinely performed to generate large numbers of shoots for transformation experiments.

Laboratory Rearing of Pink Corn Borer

There is a need to develop insect colonies of *Sesamia cretica*

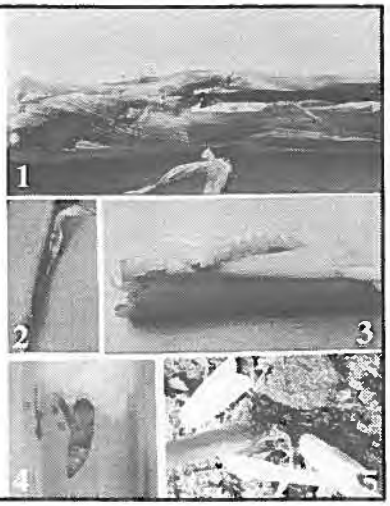


Fig. 5. (1) A corn stem infested with pink corn borer *S. cretica*, (2) eggs, (3) larva, (4) pupa, (5) moths.

in Egypt to be able to carry out laboratory bioassay and field testing.

Different semi-artificial diets have been used for laboratory rearing of the insect, but emerged adults failed to produce fertilized eggs.

Therefore a con-ventional method for rearing has been used where larvae are fed on corn stems in vials until pupation. Pupae are sexed and each couple (male and female) is kept in a vial until mating and egg laying (Fig. 5).

Laboratory Bioassay

Bt protein from C12, an Egyptian *Bacillus thruingiensis* isolate, was incorporated in corn stalks via capillary action and infested with 1st instar larvae of *Sesamia cretica*. This bioassay was developed at AGERI and is shown in Fig. 6; mortality depends on Bt concentrations that were used.

Material Transfer

1. MSU has provided to AGERI plasmids pTW-a (pin2 and BAR gene) and pRAC-15 (GUS gene) in addition to a full length cDDA clone encoding chitinase.
2. Egyptian maize germplasm was sent to MSU.

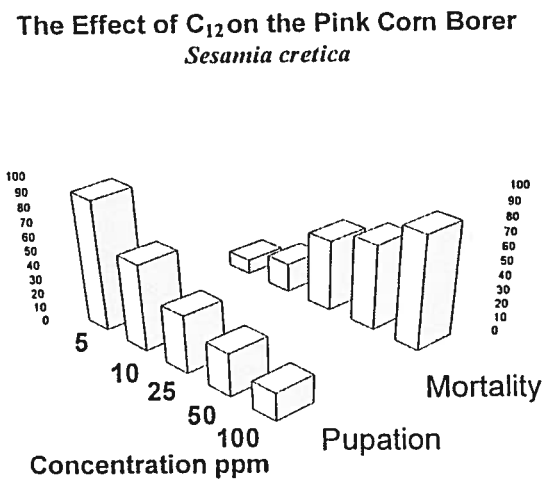


Fig. 6. The effect of Bt protein from C12 an Egyptian *B. thuringiensis* isolate.

Potato Transformation for Development of Tuber Moth Resistance - U.S.

David S. Douches
Edward J. Grafius
Walter L. Pett
Michigan State University

Objectives

The overall objective of the project is to train scientists from Egypt and Indonesia to genetically engineer the cultivated potato, conduct field trials under federal biosafety guidelines, evaluate potato tuber moth (*Phthorimaea operculella*) resistance under field and laboratory environments, and develop breeding schemes to efficiently use and maintain the effectiveness of transgenic potato lines in potato variety development efforts.

Rationale

To achieve the above stated objectives we have genetically modified Egyptian and U.S. potato lines to express *Bacillus thuringiensis* (Bt) crystalline proteins which render these lines insect resistant. These transgenic lines were compared to nontransformed lines for potato tuber moth control under laboratory and field studies. We have also tested potato lines from North Dakota State University, U.S. Department of Agriculture (USDA), and New Mexico State University which express various insect resistant genes, or have natural insect resistant mechanisms. Using different Bt constructs with various gene promoters (i.e.. leaf expression or tuber-specific expression), and combining these with lines that have natural insect resistance, we hope to develop sustainable insect resistant potato lines.

Research Achievements

Potato Transformation

Initial transformation studies were conducted to identify a genotype-independent transformation/regeneration system for potato. These studies were conducted using both the pWB139-Bt construct and the PVYcp construct. Ten different cultivars and breeding lines were used in the transformation experiment. Four different transformation/regeneration systems were examined. Transgenic shoots were obtained from all plant materials used. FL1607, Lemhi Russet, Atlantic and Spunta were identified as potato lines which are most easily transformed. The Yadav and Sticklen (1995) protocol was determined to be the most genotype-independent system in our lab.

The initial Bt construct (pWB139-Bt) was obtained from Dr. Wayne Barnes, Washington University. The gene is a wild type CryIA(c) Bt with moderate expression levels in potato. Two transgenic lines expressing the wild type Bt have been greenhouse-tested and are currently being field tested in Egypt. A second Bt gene was obtained from ICI Seeds. This Cry V-Bt gene, with both lepidopteran and coleopteran activity, has been codon-modified to increase gene expression levels. Vector constructs have been made with the Cry V-Bt gene as follows:

<u>vectors with GUS</u>

35sCaMV/Cry V
gelvin superpromoter/Cry V
patatin/Cry V

vectors without GUS

35sCaMV/Cry V

gelvin superpromoter/Cry V

35sCaMV/ Cry V+ PVYcp

A patatin/Cry V - GUS vector is currently being constructed.

The bulk of the transformation research since obtaining the Cry V-Bt gene has used the 35sCaMV/Cry V + GUS vector. Three genotypes were used for transformation: Lemhi Russet, USDA80-1 (high leptine) and L235-4 (glandular trichomes). The purpose of this research direction was to combine natural (high leptine and grandular trichomes) and engineered resistance to potato tuber moth. Numerous confirmed (PCR-positive) transgenic lines were obtained for each of the three genotypes. These lines have been extensively tested in potato tuber moth feeding studies and a subsample was chosen for Northern and Southern analyses. Some of these lines have been distributed to Egypt as tissue culture plantlets to initiate greenhouse evaluations and future field studies.

Our next focus has been to conduct transformations to evaluate the other Cry V vectors that have been constructed. Using Spunta, a cultivar adapted to Egypt climate and potato production, numerous trans-formations have been conducted. Transgenic lines have been recovered from the various constructs (35sCaMV/Cry V + GUS, gelvin superpromoter/ Cry V + GUS, patatin/Cry V + GUS, 35sCaMV/ Cry V - GUS). Both PCR analysis and Southern analysis confirm the integration of these constructs. Northern analysis confirmed the tuber-specific expression of the Cry V-Bt when combined with the patatin promoter, while the gelvin superpromoter and 35sCaMV promoter resulted in constituitively expressed CryV-Bt.

Laboratory Evaluation of Potato Tuber Moth Resistance

To date over 120 transgenic potato lines have been tested in our laboratory using our leaf feeding bioassay. Each replication of the bioassay consists of placing 5 newly hatched potato tuber moth first instar larvae on excised potato leaves. The leaf petioles were kept in water filled vials sealed with parafilm and the leaves remained turgid for 6 days. Insect mortality was recorded 48 and 72 hours after the insects were placed on the leaves.

Our latest test examined potato tuber moth control of Spunta lines that were transformed with the following constructs:

vectors with GUS

35sCaMV/Cry V

gelvin superpromoter/Cry V

patatin/Cry V

vectors without GUS

35sCaMV/Cry V

Excellent control was observed with the GUS minus transgenics; all larvae died and little feeding occurred.

Tuber feeding studies were used to determine the control levels of tubers against

potato tuber moth. Five newly hatched potato tuber moth larvae were placed on tubers kept in plastic cottage cheese containers. The tubers are checked for number of larvae, pupae and adults 4 weeks after infestation. We are currently testing the Spunta transgenics listed above. Plant lines that provide greater than 90% control of potato tuber moth in laboratory studies will be used in field trials.

Field Trials

Field trials testing insect control and the agronomic qualities of transgenic and nontransformed potatoes have been conducted yearly, since 1993, at the Montcalm Potato Farm, Montcalm County, Michigan. The experiments were planted in a randomized complete block design replicated six times. Treatments (plant lines) over the past four years have included 29 lines from 7 different potato cultivars (Table 1 page 36). Tobacco hornworm (*Manduca sexta*) larvae were used as our test insect because potato tuber moth are not indigenous to Michigan. Three or five first instar tobacco hornworm larvae per plant in each treatment were observed for mortality.

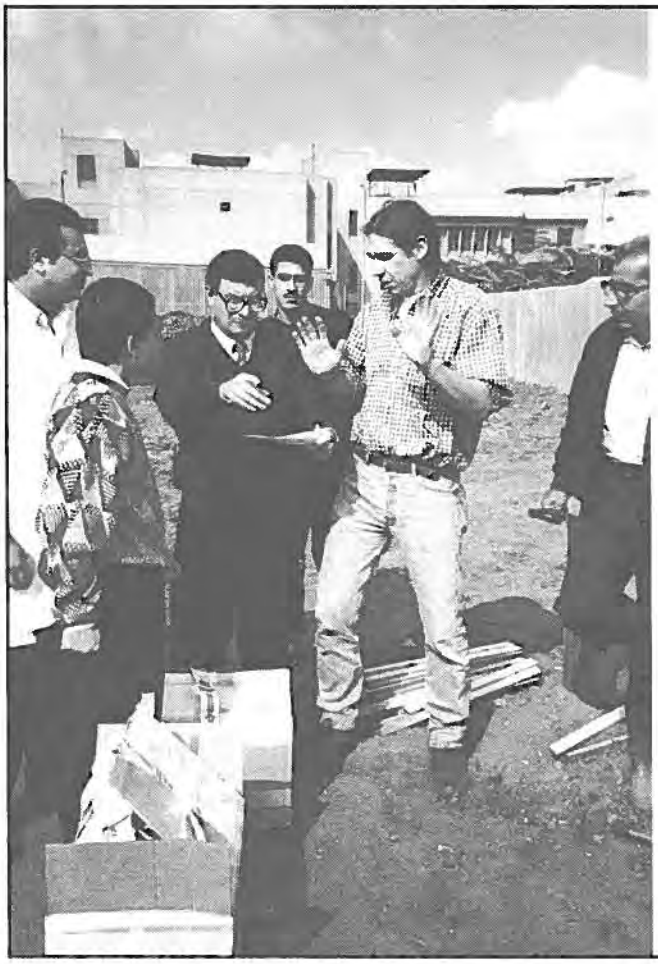
Agronomic qualities, including yield data and tuber size, were collected at harvest. Tubers from lines that showed the best insect control, and yielded high agronomic qualities were saved for future field trials. Lines that did not perform up to standard were discarded.

Field trials to maximize potato tuber moth infestations were conducted at the University of California-Davis, Davis, California, in 1996. These trials included lines that provided excellent potato tuber moth control in the laboratory and

AGERI field of transgenic potato plants. January 1997.

showed high agronomic qualities in Michigan field trials. Potato tuber moth adults were introduced to all treatment lines and were observed for larval feeding. Unfortunately, high temperatures destroyed all potato tuber moth adults before egg laying began. Yield from the harvested plots were comparable to Michigan yields.

Our current field study was planted January 21, 1997, at the Agricultural Genetic Engineering Research Institute (AGERI) in Giza, Egypt. Tubers of ten transgenic potato lines and three nontransformed control lines were planted in a complete randomized block design replicated four times. Potato tuber moth pupae will be introduced to the treatment lines 6 to 7 weeks after the plants emerge. The number of leaf mines and defoliation rating will be taken weekly. Yield and agronomic qualities will be determined at harvest.



Dr. Walter Pett (Michigan State University) and Dr. Taymour Nasr El-Din (Agricultural Genetic Engineering Research Institute) planning the planting of transgenic potatoes at AGERI, Egypt. January 1997.

Table 1.

Year and location of field test using transgenic and nontransformed potato lines that were conducted under the Agricultural Biotechnology for Sustainable Productivity Project.

Line	Resistance Factor	Year				
		1993	1994	1995	1996	1997
FL 1607		MI	MI	MI	MI, CA	Egypt
FL 1607 Bt	CryIA(c)[1]	MI	MI			
FL 1607 Bt-9	"			MI		
FL 1607 Bt-11	"			MI	MI, CA	Egypt
FL 1607 Bt-30	"			MI	MI, CA	
FL 1607 Bt-35	"			MI	MI, CA	Egypt
Atlantic		MI	MI		MI	Egypt
Atl Bt 1	CryV[2]				MI	Egypt
Atl Bt 5	"				MI	Egypt
Atl Bt 7	"				MI	Egypt
Atl Bt 8	"				MI	Egypt
Lemhi Russet				MI	MI, CA	Egypt
LR346.5.1	CryIA(c)[3]			MI	MI	
LR346.13.1	"				MI, CA	
LR346.14.1	"				MI, CA	
LR346.15.1	"			MI	MI, CA	Egypt
LR346.16.1	"				MI, CA	
LR346.20.1	"				MI, CA	
LR346.21.1	"				MI, CA	Egypt
LR346.24.1	"				MI, CA	Egypt
L235-4	Glandular trichomes (GT)[4]			MI	MI, CA	Egypt
L235-4 Bt2	GT plus CryIA(c)[1]			MI		
L235-4 Bt9	"			MI		
Russet Burbank				MI		
RBI346.7.1	CryIA(c)[3]			MI		
RBI346.10.1	"			MI		
RBI346.17.1	"			MI		
KWPTM 24	Natural resistance[5]			MI	MI	
USDA80-1	Leptines[6]			MI	MI	

[1]Wildtype Bt construct provided by W. Barnes, Washington University, St. Louis, Missouri.
[2]Codon modified Bt construct provided by ICI Seeds, Ames, Iowa.
[3]Codon modified Bt construct provided by C. Brown, USDA, Prosser, Washington.
[4]Provided by Cornell Univesity, New York.
[5]Provided by International Potato Center, Lima, Peru.
[6]Provided by USDA, Beltsville, Maryland.

Meetings

U.S./Egypt Biosafety Workshop, Cairo, Egypt, January 1994

USDA Potato Research Workshop, Calgary, Canada, July 1994

Potato Association of America, Calgary, Canada, July 1994

Entomology Society of America, Dallas, TX,U.S., December 1994

Pest Resistance Workshop, Montevideo, Uruguay, March 1995

Potato Association of America, Bangor, Maine, U.S., July 1995

Entomology Society of America, Las Vegas, Nevada, U.S., December 1995

Potato Association of America, Twin Falls, Idaho, U.S., August 1996

10th Biannual Conference on Pesticides, Huang-Shan, China, October 1996

Entomology Society of America, Louisville, Kentucky, U.S., December 1996

Development of Potato Tuber Moth Resistance in Potato - Egypt

Magdy Madkour
Taymour Nasr El-Din
Mamdouh Idriss
Agricultural Genetic Engineering Research Institute

Ramzy El-Bedewey
International Potato Center

Introduction

Potato (*Solanum tuberosum* L.) is an important vegetable crop in Egypt. The area of potato under production has reached 292,000 hectare/year over three seasons (i.e. winter, spring, and summer). The total production was 2.5 million tons in 1995 with the winter season crop used mainly for export. Last year Egypt exported 435,000 tons to Europe and the Arab countries. The yield is affected by infestation with potato tuber moth (PTM) *Phthorimaea operculella* (Zeller). The insect attacks potato plants in two ways: by mining the foliage and feeding on tubers as well. Therefore, it is an important pest both in field and storage.

Objectives

1. Characterization, isolation and cloning of Bt endotoxin genes from Egyptian isolates of *Bacillus thuringienseis*.
2. Develop and standardize bioassay procedure for testing Bt varieties against PTM and other insect pests.
3. Improve regeneration and transformation system for Egyptian potato cultivars.
4. Evaluate Bt-transgenic lines in the greenhouse and field.

Potato Regeneration and Transformation

To improve the regeneration system for the most common Egyptian potato cultivars, some basic experiments were conducted through this project. The experiments were based on methods published for regeneration of tetraploid and diploid potato lines.

Potato Micropropagation

In vitro potato plantlets of *Solanum tubersoum* tetraploid cultivars (namely Spunta, Desiree and Cara) were propagated by nodal cutting technique. MS medium used as a basal medium supplemented with 2% sucrose, 2.25 mg/1 GA_3 and solidified with 2 g/1 phytagel.

Because of using *in vitro* leaflets in regeneration and transformation systems, another modification was carried out on MS medium used for micropropagation of potato. Silver thiosulfate solution (0.1 M, STS) consisting of silver nitrate and sodium thiosulfate was added to MS potato nodal cutting medium. The experiment was implemented to increase the surface leaf area and enhance the leaf quality. The addition of STS enhanced the *in vitro* plantlet growth dramatically, enlarged the leaflet area and increased the weight and number of leaflets. The best concentration of STS was 1.5 ml/liter incorporated with micropropagation MS medium.

Potato Regeneration

Various explants of potato were used for regeneration such as leaf discs, tuber discs, *in vitro* leaflets and internodes. Results indicated that the use of *in vitro* leaflet as explant and

cultured on modified MS medium leads to the best regeneration capacity and the highest number of proliferated shoots per leaflet (Fig. 1). The advantage of this method is to avoid contamination problems, seasonal fluctuation and decline of tuber quality. The regeneration of major potato cultivars from leaves (*in vitro* leaflets) grown on different media is illustrated in Fig. 2. The regeneration ability of upper and lower parts of *in vitro* leaflets in various cultivars is presented in Fig. 3. Results showed that the lower part is more regenerable than the upper part.

Transformation Experiments

The Bt endotoxin Cry(a) gene was isolated and identified from Egyptian bacterial strain C12 of *Bacillus thuringiensis*. The full length wild type Bt gene was cloned in a plant vector pBI 121 (Fig. 4) and introduced into *Agrobacterium tumefaciens* strain LBA4404 for plant transformation. *In vitro* potato leaflets were inoculated with the *Agrobacterium*. Transformation experiments yielded seven putatively transformed potato lines from Desiree and Spunta cultivars (Table 1) out of total 30 plantlets regenerated in the experiment.

Fig. 1. In vitro regeneration of potato leaflets on modified MS regeneration medium.

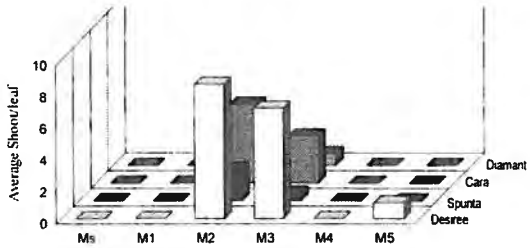


Fig. 2 Regeneration of different potato cultivars grown on MS media supplemented with different growth regulators and complex addenda as follows.
MS=without regulators
MS1=MS+1mg/1BA+1mg/1IAA
MS2=MS+1mg/1BA+1mg/1IAA+10mg/1GA3
MS3=MS+1mg/1BA+10mg/1GA3
MS4=MS+20mg/1IAA+0.01mg/1NAA+50mg/1CW
MS5=MS+0.1mg/1NAA+10mg/1Kin

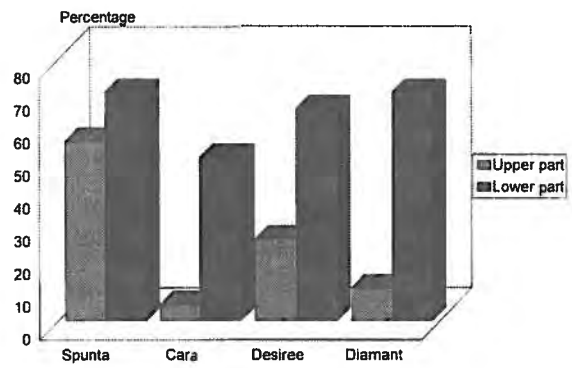


Fig. 3. Regeneration ability of upper and lower parts of the *in vitro* leaflets in various potato cultivars.

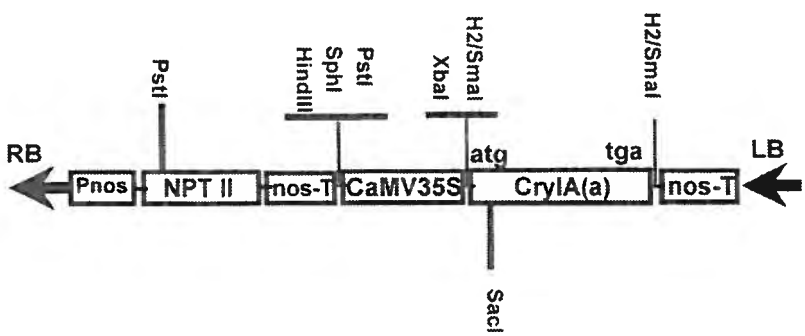


Fig. 4. Bt gene CryIA(a) cassette with CaMV 35S promotor in pBI 121.

Gene insertion was confirmed in the transgenic plants by carrying out the following tests:

- PCR: to detect the presence of Kanamycin and Bt genes.
- Southern Blotting: Bt labeled probe was used for hybridization.
- NPT II ELISA assay.
- ELISA: for detection of Bt-endotoxin product and level of production in transformed plants.

Transformed tissue was cultured on callus induction selective medium containing (100 mg/l) Kanamycin sulfate.

Transgenic potato lines were transferred to the biocontainment greenhouse (Fig. 5) for minituber production.

Development of Rapid Bioassay for PTM

Several concentrations of Bt (C12) protoxin were tested. The results of bioassay experiments to evaluate the toxicity of C12 (local strain) compared with standard Bt (K) strain revealed similar responses (Fig. 6).

Minitubers of potato Bt-MSU lines (received from D. Douches) were propagated and harvested from the biocontainment greenhouse and used in this bioassay experiment. Potato lines were infested with first instar of *P. opercullela* (PTM) larva. Each line received 25 larva and the treatment was replicated three times and included the control (non-transgenic). The experiment was conducted on tubers (ca 150 g/

Table 1

Variety	Line
Desiree	Bt E 8
Desiree	Bt E 9
Desiree	Bt E 13
Desiree	Bt E 26
Spunta	Bt E 27
Spunta	Bt E 32
Spunta	Bt E 73

treatment) in small glass jars covered with paper tissue. Jars were incubated at constant (25 C) temperature in the insectory room. The percentage of moth emergence in transgenic lines were calculated after one month and compared with control lines.

Different potato lines showed variation in their potency against potato tuber moth larvae. FL-1607 Bt 11 transgenic potato line which has endotoxin gene Cry(c) wild type was the most potent line (64% inhibition of adult formation), compared with FL-1607 control (Fig. 7). Line PI 1230502 is a wild type potato (diploid) with natural resistant genes and was used in this experiment as positive control. On the other hand, three potato lines transformed with CryIA(a) (Egyptian strain) were also tested and showed moderate resistance. The results are presented in Fig. 8.

Fig. 5. Transgenic potato lines grown in biocontainment greenhouse.

Evaluation of Bt Transgenic Lines in the Field

Field studies for testing transgenic and non-transgenic potato lines against PTM in Egypt (AGERI experimental fields) were conducted in January 1997. Potato tubers were planted in randomized complete block design with 4 replications. Treatments included different Cry genes (i.e. Cry IA(c) wild type Bt. Cry IA(c) codon modified Bt and modified CryV). The experiment is still in progress.

Material Exchanged

1. Received from MSU:
   a. Four Cry V constructs in *Agrobacterium*.
   b. Transgenic tissue culture plantlets.
   c. Transgenic tubers for field trials.
2. Sent to MSU:
   a. Bt-Gene of C12 Isolate.

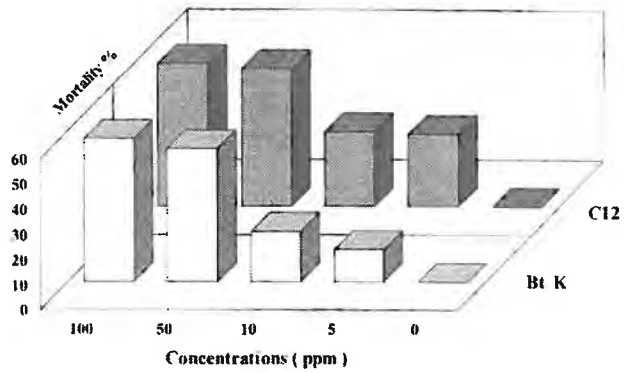


Fig. 6. Percentage of the Larvae mortality of PTM using C12 bacterial strain compared with Bt K othanitic strain.

Main Achievements

1. Isolation, identification and cloning of Bt genes in Egypt.
2. Establishment of regeneration system for some Egyptian potato cultivars.
3. Development of transgenic potato lines which confer resistance to PTM.

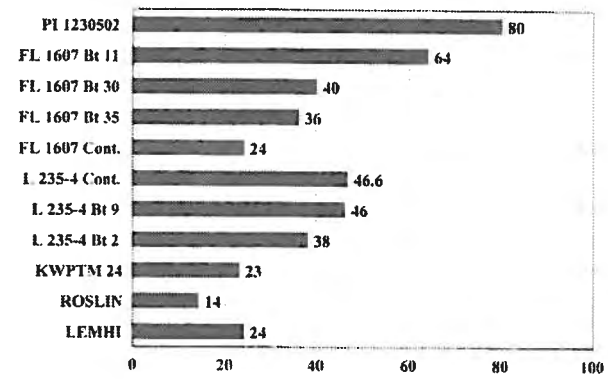


Fig. 7. Bioassay experiments for evaluation of transgenic Bt-MSU potato lines.

4. Establishment of bioassay method for PTM.
5. Conducting field tests for transgenic potato lines under biosafety guidelines.

Future Prospects

1. Field testing in different locations in Egypt with the help of private sector and CIP-Egypt to evaluate trans-genic lines under natural field condi-tions.
2. Evaluation of trans-genic tubers under local conventional storage facilities (Nawala).
3. Use the evaluated Bt-potato lines in an IPM system for potato production.

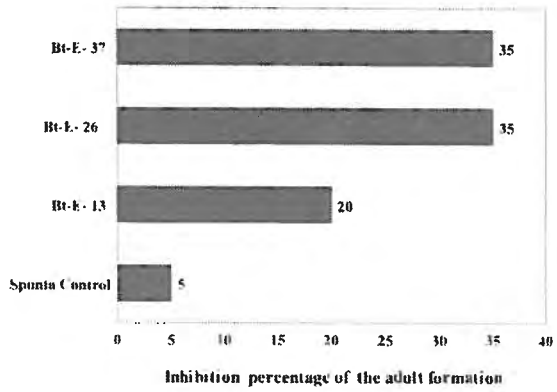


Fig. 8. Bioassay for evaluation of transgenic Egyptian Bt potato lines.

Development of Insect and Virus Resistance in Sweetpotato - U.S.

Maud Hinchee
Jeffrey Lowe
Monsanto Company

Project Goal

To engineer sweetpotato plants with increased resistance to sweetpotato weevil, and to engineer sweetpotato for resistance to sweetpotato feathery mottle virus (SPFMV).

Research Progress

The work in this report was carried out in collaboration with Mr. Daniel Maingi and Mrs. Charity Macharia, of the Kenya Agricultural Research Institute (KARI), at Monsanto Agricultural Company, St. Louis, Missouri. The purpose of this collaboration was to continue the development of a transformation system for various sweetpotato genotypes. It summarizes results obtained through March 1996, when direct support to the project concluded.

Introduction

At the inception of the project, the only reproducible report of sweetpotato transformation concerned the inoculation of storage root tissue, of the genotype Jewel, with *Agrobacterium tumefaciens* and regeneration of plants (CA Newell et al 1995) expressing cowpea trypsin inhibitor and snowdrop lectin. There have been other reports detailing the production of transgenic plants from other explants sources, such as petioles (Prakash, personal communication). However, there have been problems reproducing these results in the same labs, and when the techniques were attempted elsewhere. Consequently, the aim of this project was to use the method of Newell, et al, and see if it could be applied to other explants and other genotypes. Further, we wanted to see if it was possible to use the other techniques for transformation that have been reported.

At the start of the project it became clear that the work at Monsanto, started by Dr. Florence Wambugu from KARI, and adapted by Mr. Daniel Maingi, was also producing transgenic plants. It was hoped that by combining the project at MSU with that at Monsanto we would develop synergy which would allow us to make more rapid progress than the two projects in isolation. Although the initial aims of the projects were different, it was felt that they were also compatible. ABSP was interested in developing sweetpotato germplasm that had increased resistance to the sweetpotato weevil for use in Indonesia, and Monsanto was focused on resistance to the sweetpotato feathery mottle virus (SPFMV) in Kenyan lines.

Embryogenesis and Regeneration

The first year of the project was undertaken at MSU (March 1994-March 1995) in the laboratory of Dr. Mariam Sticklen, where it was not possible for Dr. Jeff Lowe and Dr. M. Herman to reproduce her results concerning embryogenesis from root tissue. At the beginning

of the second year (March 1995-January 1996), Dr. Lowe moved to Monsanto in St. Louis to work with Mr. Daniel Maingi and Dr. Maud Hinchee. At Monsanto there were 8 African genotypes supplied by KARI (CPT560, Toilo, KSP203, KSP36, Nyaluolo, Olombo, Kemb33 and Kalamu nyerere), as well as 8 American lines (Jewel, Regal, Beauregard, Hi-Dry, Cherokee, Papota, White Star and 318846). While Mr. Maingi continued to adapt the system developed by Dr. Wambugu, Dr. Lowe took four main approaches to developing transgenic plants.

First, he looked at the system of Newell et al (1995), which relied upon the production of embryogenic cultures from storage root tissue. Using material obtained from a local grocery store, it was possible, with uninoculated material, to produce embryos which gave rise to plantlets. However, we experienced considerable problems producing storage roots from our genotypes of interest, and frequent contamination when pieces of tissue were placed in culture. Consequently, Dr. Lowe investigated using *in vitro* petioles, leaf pieces, and root pieces with the media sequence of Newell et al (1995). Two hundred to 800 explants per genotype were placed on the media and transferred according to the published procedure.

In order to be able to effectively compare the explants' response, a system for assessing the extent of embryogenesis was developed as follows: 0=no embryos, 1=1 to a few embryos, 2=several embryos, 3=many embryos, 4=most of the explant had embryos, 5=explant appeared to be covered in embryos. After 8-10 weeks, and 16-18 weeks, the cultures were given an embryo rating (0-5), and the percentage (0-100) of explants responding was determined. By multiplying the two figures together (0-5 x 0-100) it was possible to analyze the data. A figure of 500 was maximal (100% of the explants were covered in embryos). It should be noted that the cultures did not follow the pattern of classical somatic embryogenesis. Rather, they developed irregularly in that they appeared to be globular embryos prior to developing a meristematic shoot tip.

The most responsive African genotypes were CPT560 (petioles, leaves and roots), Kalamu nyerere (petioles and leaves), Nyaluolo (leaves) and Olombo (leaves). The only genotype that did not form embryos was KSP36. The most responsive American genotypes were Jewel (petioles and leaves), Regal (petioles, leaves and roots), Hi-Dry (petioles and leaves) and Beauregard (petioles and leaves). What is important is that it was possible to produce embryos with at least 1 explant type with 15 of the 16 genotypes (only KSP36 was unresponsive; this genotype has been stimulated to produce embryos by Mr. Maingi), while most reports of embryogenesis only deal with one or two genotypes. Furthermore, it has been possible to regenerate plantlets from a number

of these lines. As the cultures were transferred according to the procedure of Newell et al (1995), it was felt that improvements could be made in terms of embryogenesis and conversion to plantlets by altering the timing of transfer between the different media. Information from this work was used in transformation studies that will be detailed later.

Second, Dr. Lowe tried to repeat the embryogenesis and regeneration results obtained by Mr. Maingi. It was found that it was possible to repeat his results, but that fewer genotypes were responsive, and it required a longer period in culture than using Dr. Newell's method.

Third, as Mr. Maingi's system produced some promising results, Dr. Lowe made eight adaptations to his stage 2 media. It was found that a number of genotypes produced embryos, but still more slowly than Dr. Newell's method.

Fourth, all 16 lines were tested with the system developed by Dr. Prakash (personal communication). Only four lines produced embryos (Kalamu nyerere, Beauregard, Cherokee and [Red] Jewel) and this occurred at very low frequency. The genotype 318846, which Dr. Prakash had most success with, did not produce any embryos.

Transformation

While Mr. Maingi was doing transformation experiments using his system, Dr. Lowe investigated using Dr. Newell's media sequence, while using information obtained from Mr. Maingi concerning his method for optimizing transformation efficiency. The main *Agrobacterium*-based construct used contained *npms* uidA and the SPFMV coat protein gene. Using Dr. Newell's stage 1 media with 1/10th MS salts + MES, supplemented with acetosyringone and galacturonic acid, it was possible to get very high levels of transformation (as visualized by adding X-Gluc after 3 and 6 weeks in culture and observing blue spots) by following the wounding, inoculation and cocultivation procedures of Mr. Maingi. Transformed tissue was selected for by the addition of 75mg/l of kanamycin, or 25-30mg/l of paromomycin, and blue spots were observed with all 6 genotypes tested (CPT560, Kalamu nyerere, Olombo, Jewel, Regal, and Hi-Dry). Presently, these cultures are being maintained on stage 2 selection medium, and transferred at 3 weekly intervals. Embryos are beginning to develop on the transformed tissue, and it is hoped that these will develop into transgenic plantlets in the near future.

Mr. Maingi trained Mrs. Charity Macharia from KARI, to demonstrate the reproducibility of his system, and she has successfully produced a transgenic plant.

Conclusions

Mr. Maingi has produced around 60 transgenic sweetpotato plants, from a number

of genotypes. Furthermore, Mrs. Macharia has been able to repeat his results, demonstrating the reproducibility of the system. Mrs. Macharia will be returning to KARI to help ensure that the technology is transferred back to Kenya, as shewill help set up a biotechnology laboratory there. The transgenic plants they have produced in the U.S. will be tested at Monsanto for increased resistance to SPFMV, and it is envisaged that any resistant plants will be sent to Kenya for field trials. It is hoped that such material, as well as any germplasm generated in Kenya, would then be made available to Indonesia, and vice versa.

It is hoped that the method developed by Dr. Lowe will be utilized by Dr. Herman in Indonesia. As the transformed tissues are now producing embryos, it is possible that transgenic plants will be produced and so there may be little need for further development. Dr. Lowe will make himself available as a consultant in the event there are any problems repeating these results in Indonesia or Kenya. It is also hoped that it may be possible for Dr. Herman to use Mr. Maingi's procedure.

Developing Virus Resistant Cucurbits - U.S.

Rebecca Grumet
Michigan State University

Molly Kyle
Rosario Provvidenti
Cornell University

Objectives and Rationale

Cucurbit crops (melons, squashes, cucumbers, watermelons) are subject to severe losses due to infection by several viruses including cucumber mosaic virus (CMV), squash mosaic virus (SqMV) and the potyviruses zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). The research groups participating in this project utilized a combination of genetic engineering and conventional breeding approaches to develop virus resistant cucurbit crops. Funding of the cucurbit subproject largely began in 1993 with the initiation of the collaboration with Egypt.

Research Achievements in the Grumet Laboratory

At the outset of the project, the coat protein gene (CP) of the Connecticut (Ct) strain of ZYMV had been cloned, engineered for expression and introduced into muskmelon plants cv. Hale's Best Jumbo. We then verified that the introduced gene was transcriptionally and translationally functional *in vivo*, and demonstrated that the transgenic regenerants and their progeny were highly resistant to infection by ZYMV in the greenhouse (Fang and Grumet, 1993). There was no symptom development or virus accumulation during the three month observation periods of the experiments. Dr. Provvidenti tested these materials against an array of ZYMV isolates, including an Egyptian isolate, and found them to be very highly resistant.

Further genetic and cytological examination of these original, highly-resistant lines, revealed that they were tetraploid rather than diploid (Yadav and Grumet, 1994). In an effort to reduce the frequency of tetraploidy we attempted regeneration using alternate explant sources and were able to develop a highly efficient system utilizing leaf explants (Yadav et al. 1996). Although tetraploids also can occur with this system, we have been able to use the leaf regeneration system successfully for transformation experiments (Fig. 1). It has been particularly useful for material with limited seed supply, e.g. multiple virus resistant material that was supplied to us from Dr. Kyle's breeding program.

A priority of the project was to introduce the gene into cultivars thought to be more suitable for production conditions in Egypt. These included the American cultivar Topmark, a line from Dr. Kyle's program, and the Egyptian cultivars Ananas El Dokki and Shahd El Dokki. In each case we produced verified transgenic individuals that were self-pollinated to produce R_1 progeny and tested for response to viral inoculation. Promising lines were carried forward for screening the R_2 and R_3 generations (Fig. 2).

Fig. 1. Melon transformation using the leaf regeneration system. Left: control; right: transformed.

Diploid transgenics of these cultivars, as well as diploid transgenics of Hale's Best Jumbo, have not demonstrated the near immunity observed in the tetraploids. The diploids do, however, appear to be more resistant than their respective parental genotypes, as manifested as a delay in symptom development, or escape from infection at lower inoculum concentrations (Fig. 3, Fig. 4) (Grumet et al. 1995).

Fig. 2. Screening transgenic melon seedlings in the greenhouse.

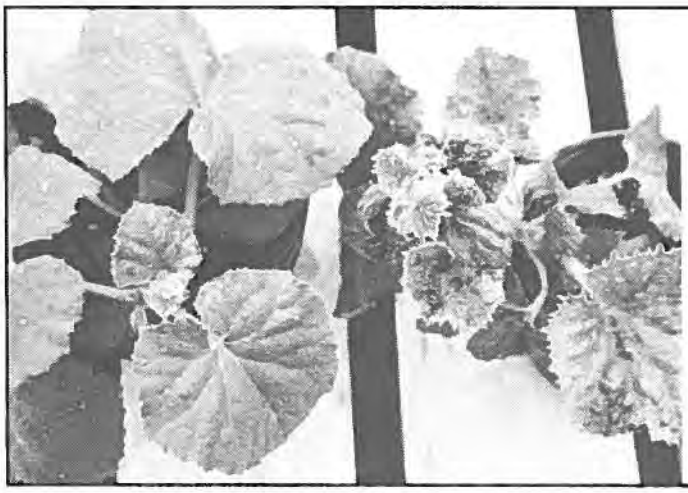


Fig. 3. Transgenic melon plant resistant to infection by ZYMV. Left: control; right: transgenic.

Transgenic materials also were

Fig. 4. A. Response of segregating progeny to inoculation by different concentrations of ZYMV. B. Response of segregating progeny to inoculation by ZYMV and WMV.

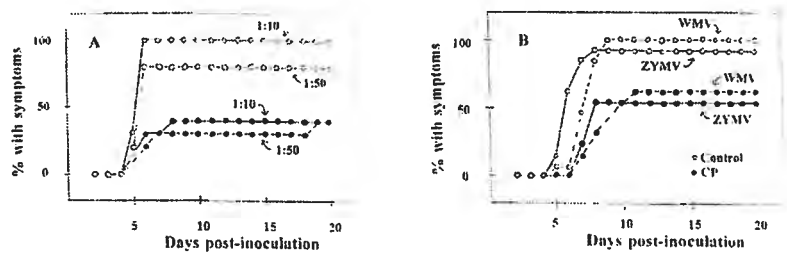


Fig. 5. Field trial of transgenic melon lines.

tested for resistance to the cucurbit potyviruses WMV and PRSV-W. Lines segregating 1:1 for ZYMV resistance also segregated 1:1 for delay in symptom development following inoculation with WMV (Fig. 4) (Grumet et al. 1995). They were not resistant to PRSV-W which is less closely related to ZYMV than is WMV. Field trials with R_2 and R_3 generation materials (Fig. 5) indicated families with segregating progeny that exhibited long delays in infection (>45 days) or did not become infected. Yield reductions of the parental genotypes were 35-60% vs. 0-22% for the best transgenic lines.

Initially it appeared that we would form a collaboration with Morocco to work with Moroccan watermelon mosaic virus (MWMV), a poorly characterized potyvirus of importance in Africa. The relationship of MWMV to the other cucurbit potyviruses had not been clear. We purified MWMV, produced antibody to the coat protein, isolated viral RNA and produced cDNAs. Sequence analysis of the CP gene provided clear demonstration that MWMV is a unique potyvirus, and that it is more closely related to PRSV-W (73% amino acid identity within the conserved core portion of the protein) than to ZYMV or WMV (61% identity).

We also identified a source of naturally occurring resistance to MWMV in cucumber. The multiple potyvirus resistant Chinese line TMG-1

was found to possess a single recessive gene conferring resistance to MWMV (Kabelka and Grumet, 1997). The gene for MWMV resistance completely co-segregates with resistance to ZYMV. Progeny analysis has indicated that a single gene or tightly linked cluster of genes in TMG confers resistance to at least four cucurbit potyviruses.

This past year, in response to interest by Indonesia and Egypt, we initiated transformation efforts with cucumber. Although our laboratory had worked with cucumber several years ago (Hammar and Grumet, 1990), we had not worked with these species during the duration of the ABSP project. In addition to resuming efforts with *Agrobacterium* mediated transformation using cotyledon explants and regeneration via somatic embryogenesis, we initiated experimentation in collaboration with Dr. Richard Allison (Michigan State University) using a direct transformation method. At this time, very preliminary results suggest a 10% success rate, which would be excellent for further work with this species.

An additional project for which the ABSP program contributed a limited portion of funding, was directed toward risk assessment due to transgenic pollen flow from cucurbits. Our studies focused on two questions: can plantings of border rows effectively limit pollen mediated gene movement, and does pollen-mediated dispersal of transgenes differ from native genes? Pollen movement within the trap borders followed a leptokurtic distribution. As the trap/donor ratio increased, there was a significant decrease in long distance gene movement to satellite plots (Hokanson et al. 1997a). Short and long distance gene dispersal data validated the assumption that native and transgenes have the same dispersal patterns (Hokanson et al. 1997b). Reviewers' responses reflected our intended goal: "although almost anyone in the field would have predicted that pollen containing a transgene would have the same pattern of dispersal as non-engineered...this is a well done study that proves a common assumption in a clear and definite way...for everyone's benefit it is very important that this issue be seriously addressed and that regulatory guidelines be developed on fact rather than assumption."

Research Achievements in the Provvidenti Laboratory

Our surveys conducted in vegetable growing areas of Egypt clearly demonstrated the widespread occurrence of squash mosaic virus (SqMV) in Cucurbitaceae, particularly in melons (*Cucumis melo*) and squashes (*Cucurbita pepo*). In nature this virus is seed transmitted and easily spread by beetles. Considering that SqMV can significantly reduce the quantity and quality of these crops, our research was directed toward locating sources of resistance which eventually

can be introduced into commercial cultivars growing in Egypt.

Squash

Initially our screening for viral resistance was directed toward commercial squash cultivars (including Egyptian cultivars), land races and wild relatives of *Cucurbita* species using a major strain of SqMV. All this material was found to be susceptible, except a wild squash from Ecuador, *Cucurbita ecuadorensis.* This species offered a tolerance to the virus, but difficulties were encountered in transferring it into cultivated squashes. A very high level of resistance was found in *Lagenaria siceraria* (bottle gourd) that is commonly cultivated in Egypt. However, all attempts to cross this species with any of the *Cucurbita* species was without success. Considering the economic importance of SqMV in cucurbits and lack of natural resistance, the concept of pathogen-derived resistance was applied in controlling this virus. Seeds of a summer squash (*C. pepo*) R_1 line possessing the coat protein (CP) gene of a melon strain of SqMV were obtained from the Biotechnological Laboratories of Cornell University (Geneva Campus) and Seminis Vegetable Seeds (Asgrow). Plants of R_2 and R_3 generations segregated for resistance to the virus, but several R_4 plants were homozygous resistant. The most vigorous of these plants were crossed with those of a SqMV-susceptible summer squash cultivar, and the resulting F_2, F_2 and reciprocal backcross populations clearly demonstrated that the resistance to SqMV was conferred by a single recessive transgene. The transgenic SqMV-resistant material also was found to be resistant to other strains belonging to the melon type (SqMV-M) and squash type (SqMV-Sq) that were originally collected in Egypt, Israel, China and the USA. From these results it was evident that the resistance obtained from the CP of SqMV is very promising in controlling the pertinent virus.

Melon

All the attempts to find sources of resistance in *Cucumis melo* germplasm from many areas of the world were unsuccessful, and the development of transgenic lines containing the coat protein (CP) gene of SqMV virus has been rather slow. However, during the last year, a few plants of a melon collected in China (*C. melo* var. *makuwa*) appeared to be resistant. Extensive studies using the progenies of those Chinese plants revealed that they were infected by strains of the virus, but foliage and fruit remained symptomless. When these highly tolerant plants were crossed with those of SqMV-susceptible melons, the resulting F_1 reacted with only a very mild green mottle, but leaves remained of normal size and shape. Of particular value were the symptomless fruits which closely resembled those of the highly productive susceptible parent. Preliminary tests already

have indicated that the strain of SqMV which had provided the CP gene is not seed transmitted in this highly tolerant Chinese melon (CH-51). Plants of this melon also were tested with strains of CMV collected in Egypt, Israel, China and the USA. All the tested plants were found to be highly resistant. More tests are contemplated during the coming months using other cucurbit viruses present in Egypt such as ZYMV, WMV, PRSV-W and PRSV-P. The introduction into Egypt of viral resistant melons and squashes could significantly help to solve the economic problems experienced by many farmers.

Research Achievements in the Kyle Laboratory

Our project was to develop cucumber, melon and squash varieties suitable for Egyptian production that carried genetic resistance to CMV, WMV, PRSV, and ZYMV. This project had several components. First we have tried to confirm that the known sources of resistance hold when inoculated with Egyptian isolates; second we have transferred existing sources of resistance to the four viruses to types that should be useful to Egyptian growers. This effort has involved the transfer of individual resistances, their subsequent combination, and finally, the selection of target varieties. Finally, we have sent seed several times for field evaluation in Egypt.

Cucumber

In cucumber, the sources of resistance were available in a cultivated type of *Cucumis sativus* and our goal was to develop a Beit Alpha type with multiple resistance. We have approached this in two ways. First, we undertook a backcross program where the donor parents for resistance were North American slicer types and the recurrent parent was Beit Alpha. Segregating progenies were screened for resistance sequentially, but since resistance is recessive, this has proven a cumbersome strategy. Alternatively, we have used Beit Alpha as a donor parent for uniformly dark smooth glossy fruit with fine spines and the North American slicer as a recurrent parent for multiple resistance. This approach has had the added advantage that the slicers have resistance to a number of other diseases that are important in Egypt, and it has proven easier to transfer the characteristic Beit Alpha type, than to try to recreate the broad resistance found in the North American varieties. This seed has been provided to our Egyptian cooperators several times, but to date we have not received an evaluation of field performance in Egypt. We have shown Egyptian visitors and North American seedsmen who breed for Middle Eastern markets the type these lines produce in Ithaca, NY, and they have assured us that the type we are recovering will be acceptable to Egyptian growers and consumers.

Squash

In squash, resistance to the four viruses in *Cucurbita pepo* has been obtained by interspecific crosses from wild types of *C. martinezii* (CMV), *C. moschata* (CMV, PRV, WMV, and ZYMV), and *C. ecuadorensis* (ZYMV). Currently we are combining resistance to CMV and ZYMV with powdery mildew resistance (PMR) in zucchini types which serve as donor lines for the Eskandarany program. We have recently re-examined the *C. moschata* resistance source, "Nigerian Local," and have discovered that the high levels of CMV, PRV and WMV resistance are also conferred by single genes. We are working with material that is segregating for several resistances and just completed our first field test for PRV resistance from this source in material that has previously been tested for resistance to the other three viruses and powdery mildew. This year we will continue with both greenhouse and field screening to develop PMR Eskandarany with resistance to all four viruses. We also are developing lines that may serve as useful hybrid parents for Middle Eastern types.

Melons

In melons, we began with Egyptian types, but after extensive consultation with our cooperators and other parties familiar with trends affecting melon production in Egypt, we have changed our target type to be a soft orange fleshed sweet cantaloupe with good shipping capabilities since it is apparently preferred by Egyptian and other consumers and will also combine well with Egyptian material. We have selected the cantaloupe variety, TAMUvalde, which is a high quality melon, very well adapted to warm conditions, that carries both PMR and DMR. This melon had been selected previously by Dr. El Doweny to be of interest for the Egyptian program. To complement our breeding efforts here, our Egyptian cooperators should plan to undertake breeding their types for resistance to CMV since this is the only virus for which resistance is required in both parents to obtain sufficient levels of resistance in a hybrid. To date, we have pairwise combinations of resistance to the viruses and have hybrids available to trial in Egypt whenever our cooperators can proceed with field tests. While the levels of resistance observed in these hybrids will still not be as high as when we complete the project, we expect that by the end of the project we will be able to provide true-breeding types homozygous for all four viruses.

Summary

To summarize, we have been successful in transferring resistance to virulent isolates of all four viruses to types of squash, melon and cucumber. We are within a year or so of having single genotypes that combine resistance to three or four of these viruses, and we are very anxious to test these materials under Egyptian

field conditions. During the period of this project, we have conducted several trials of commercial and our own transgenic materials screened using the same procedures we have used for this project. In all cases, we usually see mild symptoms since we use a very severe test, followed by recovery and production of symptom-free fruit. Most companies that have generated and trialed transgene-mediated resistance to these four viruses in squash and melons feel strongly that this type of resistance, whether based on viral coat protein or replicase sequences, should be backed up with genetic resistance where available. Our materials produced under this project should be useful directly as open-pollinated varieties, as parents for hybrid varieties and in combination with transgenic resistance to one or more of the viruses.

Travel

R. Grumet, H. Munger and R. Provvidenti, January 1993, Egypt.

R. Grumet, R.C. Yadav, M. Saleh, September 1993, Cornell University, New York, USA.

H. Munger, R. Provvidenti, October 1993, Egypt.

R. Provvidenti, R.C. Yadav, January 1994, Egypt.

R.C. Yadav, 1994, Indonesia.

Development of Virus Resistance in Cucurbit Crops - Egypt

Magdy Madkour
Ahmed Abou-Salha
Mahmoud Rifaat
Hamdy El-Doweny
Agricultural Genetic Engineering Research Institute

Project Goals

1. Development of virus resistant cucurbit crops via genetic engineering and conventional breeding approaches.
2. Introduction of the ZYMV-CP gene into Egyptian melon and squash cultivars.

Research Achievements

Transformation and Regeneration of Egyptian Melon and Squash

We have introduced the ZYMV-CP gene (Connecticut strain) to the Egyptian squash cultivar Askandarani and to melon cultivars Ananas El-Dokki and Shahd El-Dokki using two different regeneration systems:

1. Regeneration via embryogenesis.
2. Regeneration via morphogenesis.

The aim of using two different regeneration systems is to study some factors which influence the efficiency of regeneration, particularly the effect of the system on the ploidy level of the transgenic plants.

Squash

Several lines of transgenic squash cultivar Askandarani were produced and verified to contain the ZYMV-CP gene by Polymerase Chain Reaction (PCR) analysis using both outer and nested primers and by ELISA test as well.

In the biocontainment greenhouse, we produced R1 seeds from the transgenic plants which were partially tolerant to viral infection.

These seeds (R1) were also grown in the open field to test them for virus resistance.

R2 seeds were produced on many transgenic squash lines and tested for response to viral infection. Some lines showed delay in the appearance of the symptoms (symptoms appeared approximately 3 weeks later than the control plants).

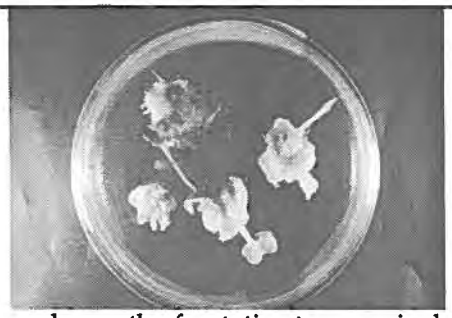


Fig. 1. Selection and growth of putative transgenic shoots of squash. Explants inoculated with Agrobacterium tumefaciens LBA4404+PCIB10 recombinant plasmid containing ZYMV-CP gene+NPT11 (kanamycin resistance) gene cultured on S3 medium supplemented with 125mg/l kanamycin and 300mg/l carbincillin.

Melon

We are continuing the transformation work with the two Egyptian melon cultivars Ananas El-Dokki and Shahd El-Dokki. Several lines of cultivar Ananas El-Dokki were produced while the response of Shahd El-Dokki was very weak.

The transgenic lines produced from Ananas were verified to contain the ZYMV-CP gene by PCR (outer and nested primers) and also by ELISA test.

We are continuing to test the transgenic plants for response to inoculation with ZYMV. Some transgenic lines were eliminated because they did not produce seeds due to the ploidy level.

R1 seeds were produced from several transgenic Ananas lines. These plants were tested against viral infection in an open field experiment and showed partial tolerance. Screening and seed production started February 1997 to develop homozygous lines with a high level of resistance.

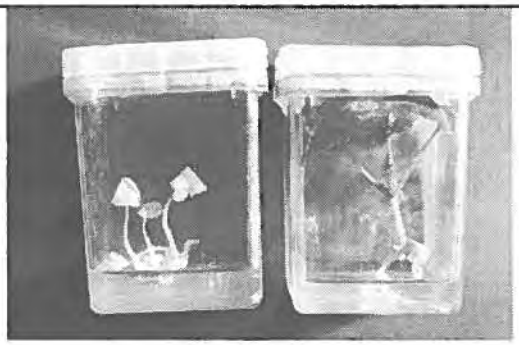


Fig. 2. Putative transgenic plantlet on MS medium without hormones with 100mg/l kamamycin. *Right,* putative transformed plantlet with ZYNV-CP gene and NPT11 (kanamycin resistant) gene. *Left,* nontransformed shoot.

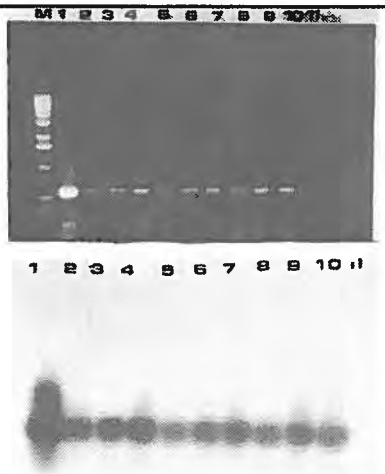


Fig. 3. A. Polymerase chain reaction (PCR) product of transgenic squash plants. Total genomic DNA was extracted from 9 transgenic plants and used as template for PCR using ZYMV-CP primers (P1 and M1). PCR products were fractionated on 1% agarose gel containing 0.5ug/ml ethidium.
B. Southern blotting hybridization by PCR products were transferred to nylon membrane and hybridized with 32P labeled ZYMV-CP probe. ZYMV-CP probe was prepared by using pCIB10 recombinant plasmid as a PCR template and ZYMV-CP specific primer bromide.
M: 1 Kb Ladder DNA marker molecular weight.
Lane 1: pCIB10 recombinant plasmid (positive control).
Lanes 2-10: different transgenic plants.
Lane 11: nontransgenic squash plants (negative control).

Fig. 4. Left, ZYMV symptoms on leaves of nontransformed squash (control). Right, resistant transgenic squash plant with full length ZYMV-CP gene.

Transgenic Tomato Project - U.S.

C. M. Fauquet

R. N. Beachy

The Scripps Research Institute

Objectives of the Project

The practical goal of the project is the production of transgenic tomatoes resistant to the tomato yellow leaf curl virus disease from Egypt (TYLCV-Eg).

Tomato yellow leaf curl disease is a very devastating disease all over the tropical countries in Africa, Middle East and Southeast Asia. The severity of the disease depends on the epidemiology of the whitefly vector, but as whiteflies invade new ecological territories, TYLCV becomes a threat in producing areas. There are virtually no efficient control methods to prevent the development of the disease: there are no suitable resistant varieties available, no agricultural techniques easy to use, and no treatments against the vector nor against the virus when the plant is infected. Tomato yellow leaf curl disease has been reported in many tropical countries in the world, especially in West Africa, East Africa, the Middle East, India, and Thailand. The disease is also now invading temperate zones and it has been recently reported in Sardinia, Sicily and Spain. The losses can be extensive and can eliminate production entirely. The disease currently prevents tomato culture in several West African countries during the winter season and causes high loss during the summer season. In Egypt, of the production of 484,963 ha, the losses have been estimated to a range of 5 to 35% depending on the season of production. Current and past agricultural practices are either ineffective or are only partially efficient, and insecticide treatments are unable to control the vector.

To reach the goal of this project, the U.S.-Egyptian research team has to study the virus causing the disease at the molecular level in order to establish a molecular strategy to control the virus, and to transfer the viral genes involved in this strategy into Egyptian tomato varieties. These tomatoes will then have to be challenged under appropriate conditions and, if positive, they would have to be evaluated by breeders for their production. Alternatively, they could be used as parental lines for transferring the gene of interest into the appropriate genotype.

Summary of Research Achievements

The stated goals of the transgenic tomato project are:

1. to establish a collaborative research project with Egyptian counterparts at AGERI (Cairo, Egypt) for exchange of information, reagents, and technology relative to the diagnosis and control of geminivirus diseases in tomato for Egypt;
2. to develop strategies and reagents for the diagnosis of such viruses for use in Egypt and other relevant regions of the world; and
3. to develop strategies via plant genetic transformation to develop transgenic plants

that are resistant to tomato yellow leaf curl virus (TYLCV), a major disease of tomatoes in Egypt.

During the course of the transgenic tomato project we have made significant progress in each of the targeted areas, as summarized.

- Several clones of TYLCV-Eg have been obtained by AGERI scientists. The sequence of the tomato clone has been done. The TYLCV-Eg isolate is a strain of the virus described in Israel and is a different virus from Italy and Spain.
- Using computer-assisted comparisons of the geminivirus database we developed oligonucleotide primers that can be used to identify whitefly-transmitted geminiviruses. The primers have been tested at ILTAB and at AGERI, and have been distributed to other colleagues in the world for field diagnosis of whitefly-transmitted geminiviruses. Many geminivirus isolates have been cloned and characterized molecularly and biologically.
- A large number of chimeric genes have been constructed using sequences derived from the genome of TYLCV-Eg. Each of the constructs were developed with the enhanced 35S promoter of cauliflower mosaic virus, and have been used in tomato transformation experiments. All of the constructs have been provided to AGERI.
- We have produced polyclonal antibodies against the coat protein, the pre-coat, and the replicase of TYLCV-Eg. These antibodies which have a high specificity and good titers, have been made available to AGERI scientists. The antibodies for the coat protein are also capable of detecting any whitefly transmitted geminivirus, as they were designed expressly for that purpose in order to be used for other geminiviruses.
- A new strategy of pathogen derived resistance for control of geminiviruses using the "pre-coat" gene of geminiviruses has been undertaken. Transient assays that were carried out gave indication that certain mutant proteins can act as dominant negative, inhibitors of disease development. A gene encoding such a dominant negative protein has been integrated into *N. benthamiana* and tomato plants: the transgenic tobacco plants have been characterized and tested for resistance, while the tomatoes are being characterized. The tobacco plants were not expressing the pre-coat protein showing a particular problem associated with this protein and therefore there was no resistant phenotype. The tomato plants will be tested as it is possible that the plant host is important for the development of such strategy.
- Another strategy of pathogen-derived resistance for control of geminiviruses using non-geminiviral proteins has been tested. Expression with a geminivirus vector of that

protein in protoplasts is leading to a drastic modification of the virus replication. When co-bombarded with a wild type geminivirus this protein prevents the virus to move systemically and the plants does not show symptoms. Transgenic tobacco and tomato plants are being made expressing this protein with a variety of constructs to increase our chances of a good level of expression for getting a resistant phenotype before the end of the current project. If these last experiments were to be successful, this strategy would lead to a universal type of control of geminiviruses, in any plant.

- Tomato transformation has been firmly established with both marker genes and genes derived from the genome of TYLCV-Eg: to date, more than 240 transgenic lines have been developed. The protocol adopted has led to frequencies of transformation approaching 9% in selected experiments. Transfer of the successful protocol to AGERI has been achieved and tomato transformation can be carried out in Egypt.
- The first transgenic tomato lines have been challenged at the F1 level with TYLCV-Eg inoculated with whiteflies, seeds have been collected for challenge at the second generation, where segregation of the transgenes will be checked. Molecular characterization of the transgenic lines is underway and should be achieved by the end of the project. The results of the challenging tests will be provided by the Egyptian team.

Research Activities

Characterizing Geminiviruses That Cause Disease in Egypt

A significant component of the research project is to document the extent of the geminivirus related diseases in Egypt. It is obvious that there are symptoms of yellow leaf curl on tomatoes in many areas of Egypt, but there are other virus-induced symptoms as well. It is not yet clear whether the sole pathogen of concern is TYLCV-Eg or other strains of geminiviruses infect tomatoes in Egypt. To determine the extent of the variability of geminiviruses (and, possibly, other virus diseases) and to select and/or develop the strategies that will be most successful we have:

1. developed a database with convenient criteria to allow one to determine if a particular virus is a strain of a previously characterized virus, or is a new virus;
2. isolated geminiviruses from various areas in Egypt that cause different disease symptoms;
3. evaluated the new virus isolates in molecular and biological characteristics;
4. determined whether or not the isolates can cause disease symptoms and yield losses comparable to those observed in the field, either alone or in combinations.

Item (1) has been completed by the U.S. team and led to the production of a diagnostic kit for geminiviruses and publication of a manuscript. Items (2) and (3) have been initiated by the Egyptian group and a lot of clones have been accumulated. The conclusions of the study are that geminiviruses are fairly homogenous in their nucleotide sequences and those sequences are most informative for comparative purposes are at the 5' end of the coat protein sequence. This finding allowed us to design specific primers to amplify sequences from essentially any whitefly transmitted geminivirus for subsequent sequence analysis. Such analyses will determine if the amplified DNA is a strain of a previously described virus or an undescribed virus. Primers have been provided to the Egyptian team for their use with cloned DNAs and collected plant samples. Primers also have been supplied to other research groups in the world, including Mexico, Arizona, and India. After gathering the information obtained by these groups this kit cold be made available on a large scale.

Gene Constructs Containing TYLCV-Eg Sequences

A variety of chimeric gene constructions have been made with sequences from TYLCV-Eg, including:

- CP (AV1) sequences in the sense and antisense orientations;
- precoat (AV2) and CP (AV1) sequences in the sense and antisense orientations;
- precoat (AV2) sequences in the sense and antisense orientations, mutated or not;
- replicase (AC1) in sense and antisense orientations; and
- replicase (AC1) in which the NTP binding site is mutated.

In each case the gene sequence was placed under control of the CaMV 35S promoter in an intermediate plasmid, and transferred to *Agrobacterium tumefaciens* for plant transformation. The AC1 mutated strategy has proven to be capable of producing resistant transgenic tobacco plants with another geminivirus and therefore there are good reasons to believe that it will work for TYLCV as well, although this remains to be shown in tomatoes. The plants have been produced and soon will be transferred to Egypt for challenging.

Product of TYLCV Specific Antibodies and Geminivirus Genetic Antibodies

ILTAB previously agreed to develop and test antibodies against the pre-coat, coat protein, and replicase genes using proteins expressed in *E. coli* and specific oligopeptides. Rabbits have been injected with expressed pre-coat and coat proteins, and serological tests demonstrated specific antibody-antigen reactions in western blots. We have never been able to express the replicase in *E. coli;* therefore, no antibodies were produced. Antibodies have also been produced with oligopeptides for the coat protein and the

replicase, the first ones are available and working in both western and ELISA. Antibodies raised against the replicase work only in ELISA and not in western tests.

Transformation of Tomatoes

Since the first year of the project, we had excellent results in our tomato transformation experiments and we produced a total of 267 transgenic tomato lines. Some of these plants contained the TYLCV-derived gene of interest and have produced seeds. The first seeds available have been transmitted to Egypt after preliminary characterization completed at ILTAB. Improvements in the handling of explants, improved transformation protocols, and experience led to more routine transformation of UC82B, and transfer of relevant technologies to Egypt.

Transformation experiments initiated at ILTAB with Dr. El-Bakry and M. El-Wahed in December 1993, were repeated and/or completed with tomato cultivars that are currently in use in Egypt. These studies confirmed that transformation carried out in the presence 20 mM proline somewhat improved the efficiency of transformation (from 1.5% of explants to 3%, in several experiments). Another set of experiments using sorbitol as a carbon source showed a very high increase in transformation efficiency (6% vs 1.3% with sucrose), despite the fact that the percentage of callus formation was lower with sorbitol than with sucrose. In view of these results, additional studies are being carried out in order to come up with a publishable report in association with AGERI.

Production, Characterization and Challenge of Transgenic Tomato Lines Expressing TYLCV Genes

About 200 lines of transgenic tomatoes have been produced and are being characterized for the presence and for the level of expression of the foreign genes; this work is still underway and should be finished by the end of the current project. The characterization is being done by PCR at the first generation and by Southern, Northern and Western blots at the second generation. However, several lines, mostly from coat protein and antisense replicase constructs, have been transferred to AGERI for challenging. The plants were submitted to whitefly inoculation by putting the healthy transgenic lines in close contact with infected tomatoes covered with whiteflies. The preliminary results indicated that most of the lines were infected but some others showed a delay of infection. Seeds are being collected and the second generation of plants has been checked for the segregation. Obviously the molecular characterization of all the transgenic lines will take some time and more time will be needed for the challenging. Unfortunately, some technical problems and lack of manpower prevented us from obtaining the

mutated replicase plants in the first phase of the program.

Development of a New Strategy of Control

A new strategy was recently conceived based upon results of studies with TYLCV-Sar and ToLCV-Au that demonstrated that the precoat sequence (AV2) is involved in symptom development. Mutations in AV2 abolished symptoms, and it was proposed that this gene may be involved in long distance movement of the virus. Constructs with AV2 from TYLCV-Eg, in both sense and antisense orientations, were constructed and transgenic plants harboring such constructs are currently under analysis. To demonstrate the role of this gene in TYLCV has not yet been possible due to the poor level of infectivity of the infectious clone of TYLCV-Eg. Therefore, we have carried out such studies with another geminivirus that infects tomatoes in India, ToLCV-In. A complete study of the function of this protein has been carried by ILTAB in order to better understand the role of the precoat in the virus replication, in the movement of the ssDNA or the particles, in the virus encapsidation and in the relationship between the precoat and the coat proteins. Experiences involving extensive mutations, deletions and modifications have been done with whole plants and protoplasts. All this work should lead to at least two publications, one having been done already. Selected mutations in this ORF have demonstrated that AV2 is essential for long distance spread and accumulation of ssDNA. Transgenic tobacco plants that constitutively express this ORF, with or without appropriate mutations, have been developed and R1 progenies have been tested. The fact that tobacco plants were not expressing this protein at all, and that we know it is very stable and abundant in the infected plants, prevented us from investigating further the strategy in tobacco. Instead we repeated the work in tomato with the hope that the gene expression would be different; the plants are now being molecularly characterized and should be tested soon.

Development of a Second New Strategy of Control of all Geminiviruses

Even if we get the above strategy to work in tomatoes, it will be a very specific strategy capable of controlling only strains of TYLCV and not any other virus. The above study also led us to work on the DNA/protein interactions of the geminivirus DNA with the pre-coat protein and in the course of this study we have identified a category of viral proteins, not from geminiviruses, that have some of the pre-coat protein properties but not others. Transient experiments expressing one of these proteins in a geminivirus vector demonstrated this fact but also showed interference with virus replication. Infection experiments with this vector and the wild type virus showed a high level of reduction of virus

replication and no symptoms at all on any of the infected plants. Naturally these transient experiments have to be confirmed with transgenic plants before we could claim resistance with this new strategy. Transgenic tobacco and tomato plants are being made and the results should be obtained before the end of the current project.

Current Status of Research Collaboration with Egypt

Relations with the Egyptian scientists are excellent and the collaboration is good. At each phase of the project a plan of action has been set up and everyone is following the plans as results permit. Currently a number of protocols, primers, gene constructs, antibodies and transgenic tomato seeds have been transferred to Egypt for utilization in the project.

The U.S. partner is in charge of :

- making constructs with the pre-coat and the replicase,
- transforming the model plant UC82B with all the constructs (from US and Egypt),
- producing the transgenic plants at the R1 generation,
- setting-up a diagnostic system for geminiviruses,
- setting-up a new strategy of control with the pre-coat gene, and
- producing antibodies against pre-coat, coat and replicase with diverse techniques.

The Egyptian partner is in charge of :

- producing an infectious clone of TYLCV-Eg,
- making constructs with the coat and the AC4,
- transforming the Egyptian varieties with the antisense replicase constructs
- challenging the transgenic plants at the R2 generation,
- evaluating the variability of tomato geminiviruses in Egypt, and
- producing antibodies against virus particles.

A kit of primers to diagnostic geminiviruses has been sent to Egypt.

All the chimeric gene constructs made in the U.S. have been transferred to Egypt.

The protocol of transformation has been transferred to Egypt.

The first transgenic seeds of several tomato lines have been transferred from the U.S. to Egypt.

Constraints and Problems for Achieving the Goals

Budget

The success of this project will firstly depend on the continuity of the funding. Current funding has been extended to September 1997. The current project has been very good to set up all the tools to work on the TYLCV, but because we had to obtain everything we spent a lot of time in the process. Now that the expertise has been obtained on each side for a number of tools, we can readily exploit them for better achievement.

Biological Constraints

Geminiviruses are difficult to control by biotechnology, all of the classical strategies are not working or with some difficulty. Geminiviruses are special in many ways, so more research will be needed to accomplish their control by genetic engineering. The preliminary results obtained so far are very unexpected and very promising in that respect, but even if they are confirmed in transgenics before the end of the current project, much more research development will be necessary to transfer them to the field.

Challenge of the Transgenics

This part of the project is very crucial and very difficult to achieve; we would have appreciated having more input in a better controlled protocol. This would have required involvement of a team of Egyptian specialists to execute the challenge and possibly a breeder to take in account the plant side.

Human Resources

Dr. El-Bakry left the project in July 1994, and it is believed that a senior cell biologist should replace him. This program should have long term training in cell biology and in molecular biology to ensure continuity in the future. This has not been done because of the lack of adequate funding but should be developed in the second phase of the project.

Intellectual Properties

The different strategies that we use in this project are either covered by a patent belonging to a U.S. company, or being filed by our team. In the case of commercialization and depending on the strategy used, this will have to be sorted out except if we manage to develop our strategies.

Linkages to Crop Improvement/Commercialization

This project is too young to consider these aspects which will have to be considered at the end of the second phase.

Transformation of Tomato for Resistance to Tomato Yellow Leaf Curl Virus - Egypt

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Project Goal

The goal of this project is the production of transgenic tomatoes resistant to tomato yellow leaf curl virus disease from Egypt (TYLCV-Eg).

Geminiviruses are single-stranded DNA viruses with a geminate capsid structure. Whitefly transmitted geminiviruses (WTG) have been identified as the important group of plant viruses infecting economic crops in Egypt, including tomato, melon, okra, sugar beet, cotton, and numerous common weeds. In order to apply suitable genetic engineering tools to produce plant resistance to geminiviruses, it was necessary to develop a diagnostic tool to identify the geminiviruses in Egypt. Tomato yellow leaf curl virus was selected as it is the most devastating geminivirus in Egypt. To develop strategies to control any disease, it is essential first to understand the genetic variability of the pathogen. Therefore, the project was divided into two major steps: the diagnosis of geminiviruses in tomato and other crops and the control of geminiviruses in tomato plants.

Diagnostic Detection to Identify Geminiviruses in Egypt

A novel PCR diagnostic method for geminiviruses in infected crops has been developed by using primers based on conserved sequences for whitefly transmitted geminivirus (WTG). The amplified PCR fragments were followed by hybridization analysis and restriction mapping to perform RFLP for the viral genome. Also, sequencing a small part of the coat protein genome was conducted to identify the isolated geminivirus.

Serological analyses of the infected plants were performed by using polyclonal antibodies for TYLCV-Eg and antibodies from synthetic oligopeptides at 5' & 3' ends for the Cp-ORF, specific for WTGs (monospecific) have been used to test the presence of geminiviruses in the infected plant tissues. Also, antisera against other open reading frames (precoat protein, C4, Rep genes) have been raised to test transgenic plants.

Developing Different Strategies to Produce transgenic tomatoes resistance to TYLCV-Eg

TYLCV-Eg genome has been sequenced and aligned with other related WTG, the genome organization of TYLCV-Eg is similar to other whitefly-transmitted geminiviruses but lacks the B component. Four open reading frames (Cp, Rep, C1 and C4) of the viral genome have been cloned and expressed in *E. coli* expression vector to study its function. The main objective of this project is to produce transgenic tomatoes resistant to TYLCV-Eg, therefore a number of different constructs have been cloned in vectors pMON977 and pMON316 and transformed to

Agrobacterium tumefaciens for plant transformation:

1. Precoat protein strategies: sense, antisense and dysfunction.
2. Coat protein strategies: sense, antisense, stabilized by oligopeptide sequence and with two different leader sequences.
3. Replicase strategies: sense, antisense and dysfunction.
4. C4 strategies: sense, antisense and dysfunction.
5. Suicide cell strategy using the sense promoter of TYLCV-Eg controlling a toxic gene.

Tomato plants transgenic for some of the constructs listed above have been produced (Table 1). Work is in progress to characterize the R1 generation plants by germination in media containing kanamycin (Km) and testing the regenerated plants by PCR, Southern and Northern analysis. Some of the R1 seeds have been tested in the field and/or agroinfection and evaluation of the resistance has been recorded.

Table (1). Summarizes the Constructs Made and Plants

Plasmid	Gene of Interest	Vector	R1 Tested	R2 Tested	Cultivar
Y101	PCp+Cp[1], antisense	979	Field		UC82B
Y102	PCp+Cp[1], sense	979	Field		UC82B
Y103	PCp, antisense	979	Field		UC82B
Y104	PCp, antisense	316	Field		UC82B
Y105	PCp, sense	316	Field		UC82B
Y106	Rep, sense	316	Field		UC82B
Y107	Rep, antisense	316	Field		UC82B
Y107	Rep, antisense	316	Field	Greenhouse	CR[5] and PT[6]
Y108	TEV Rep[2], antisense	977	Field		UC82B
Y109	TEV Rep[2], sense	977	Field		UC82B
Y111	N-ter D PCp[3], sense	977	Field		UC82B
pMNCp	Cp, sense	977	Field		UC82B
pFstCP	Cp without first met	977	Field		UC82B
pMNSpp	Cp with stabilizing peptide	977	Field		UC82B

[1]Deletion of 22 a.a at the 3' end of Cp
[2]Rep fused to TEV leader
[3]PCp from ToLCV-India with deletion o 18 a.a.
[4]Replicase mutated (site directed mutagenesis).
[5]CastleRock cultivar
[6]Peto cultivar

Technology Transfer

Mohamed Abdel-Wahed has spent seven months in the ILTAB-TSRI from August 1993 to March 1994 to train on modern technology for tomato transformation and regeneration via *Agrobacterium* technology. Transformation was performed successfully with both standard and commercial tomato cultivars. The efficiency of tomato regeneration/transformation technique was higher with the commercial cultivars.

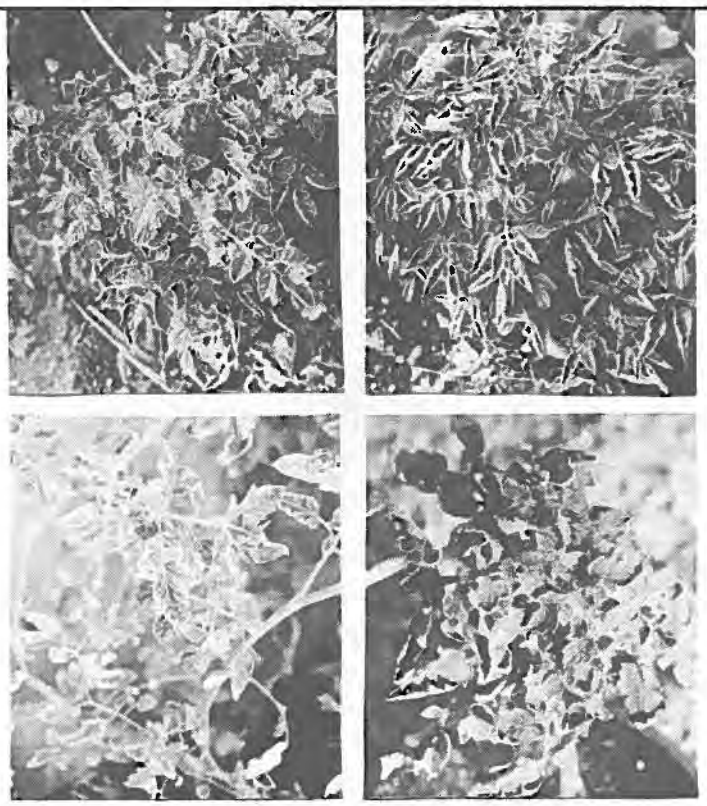

Fig. 1. Different leaf symptoms of the whitefly transmitted geminivirus on tomatoes in the field. Viruses cause: (a) stunting and curling, (b) rolling and curling, (c) curling and yellowing, and (d) rolling and leaf malformation.

The transformation system was established using a kanamycin resistant gene as a selectable marker gene and the GUS gene as a reporter gene.

After the training period the transformation system was established at AGERI using the gene of interest with the kanamycin gene as a selectable marker gene.

Transferred Materials

AGERI received antisera for coat protein, precoat and replicase protein from ILTAB. Also, transgenic seed for all the constructs in Table 1 were received except CastleRock seeds which were in AGERI.

Continued Needs for the Project

To fulfill the aim of the project, we need to perform the following:

- More field tests for the transgenic tomatoes using constructs that have not been tested to identify the best strategy for virus resistance
- Transform a commercial cultivar of Egyptian tomatoes with the best strategies to produce tomatoes resistant to TYLCV-Eg.
- Study the morphological variability and fertility in the R1 and R2 plants.

- Select the homozygote plants for the introduced gene in R2 plants.
- Test the stability of resistance in R2 plants before giving it to the end-user.

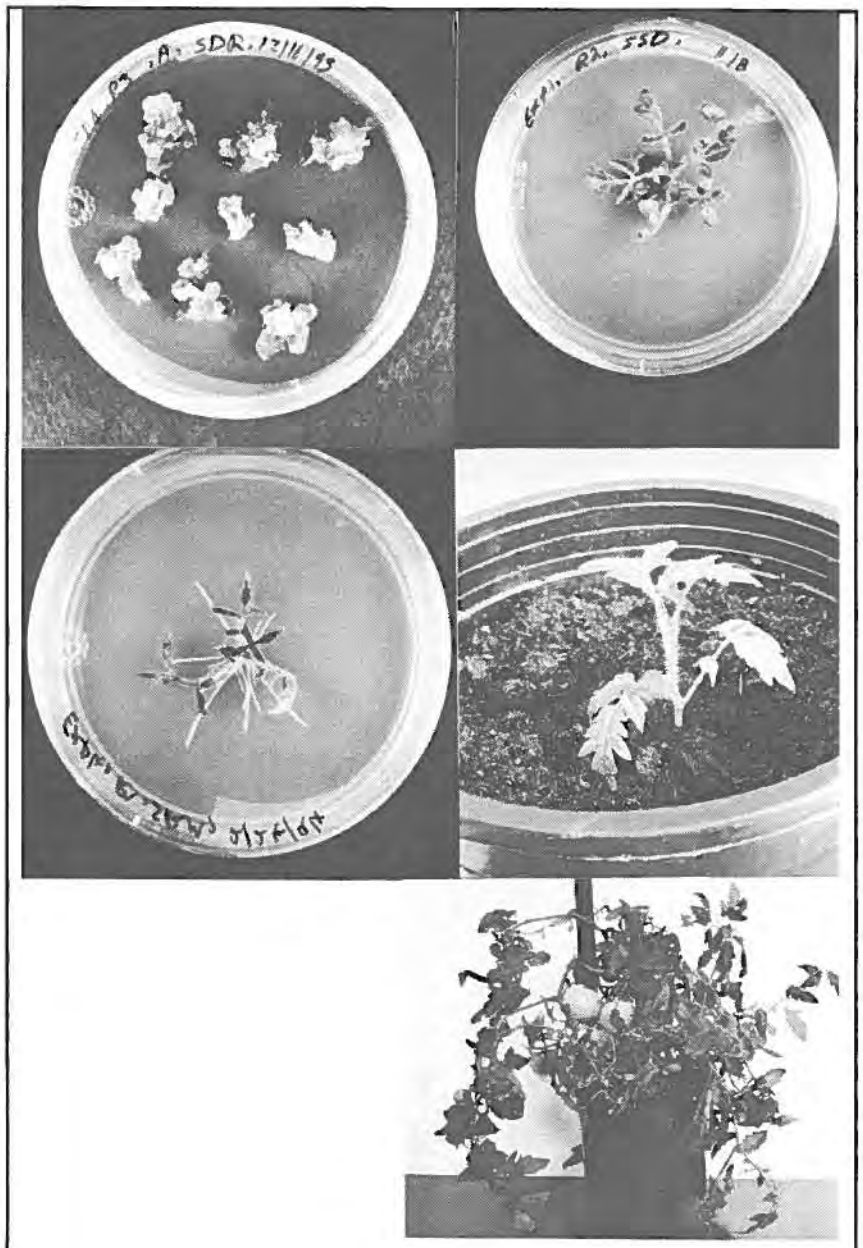

Fig. 2. Transformation and regeneration of tomatoes: (a) tomato cotyledons on selected media 3 weeks after cocultivation with Agrobacterim showing callus shooting induction, (b) shoot elongation, (c) root elongation, (d) plantlet during acclimation process, (e) well developed plant showing flowering fruit formation.

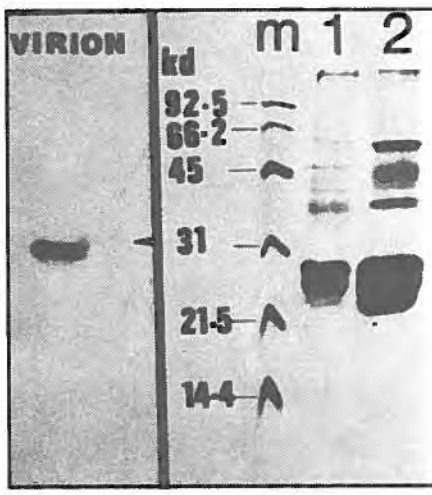

Fig. 3. Polyacrylamide gel electrophoresis analysis of TYLCV-Eg protein with partially purified (lanes 1 and 2) and sucrose density-gradient purification (verin).

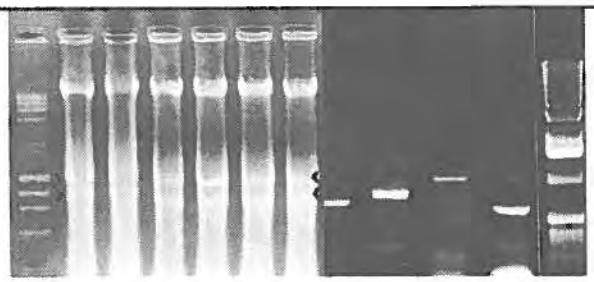

Fig. 4. Agarose gel electrophoresis (a) of total DNA extracted from infected tomatoes with TYLCV-Eg, arrows represent the DNA viral genome, (b) PCR amplification of DNA isolated from agroinfected tomatoes with TYLCV-Eg infectious clone using different primers to amplify the core-Cp, the CP-ORF, the Rep-ORF, the Rep-ORF and the core-Rep.

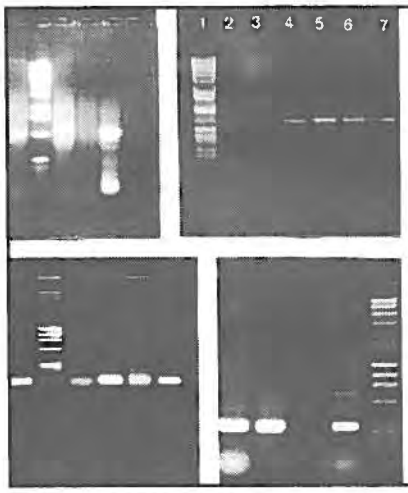

Fig. 5. PCR amplication of total DNA from infected tomatoes with whitefly transmitted geminiviruses collected from the field by using primers to flanking the Rep, the Cp, the C4 genes and the common region of the TYLCV-Eg.

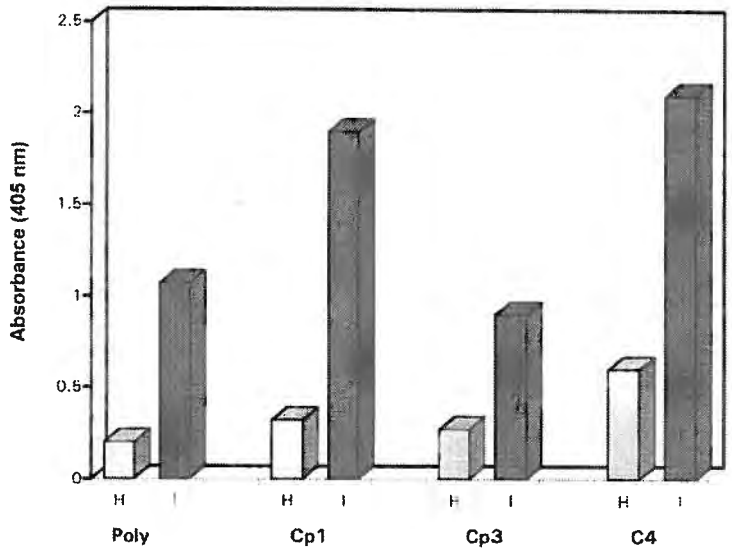

Fig. 6. Detection of TYLCV-Eg in infected tomatoes by means of Double Antibodies Sandwich Enzyme Linked Immuno-Sorbent Assays (DAS-ELISA). Means values were statistically calculated from the average absorbance of double wells for individual samples. Each column represents mean of 3 replicates either for healthy (H) or infected (I) tomatoes. Four types of antibodies were used polyclonal (Pol) against purified virus, Cp1 and Cp3 which represent antibody against Cp5′ and 3′ ends oligopeptide and C4 against E. coli expressed and purified C4 protein.

Cloning and Characterization of Insecticidal Genes from *Bacillus thuringiensis* - U.S.

Lee Bulla

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Objectives and Rationale

The objectives are to address important questions that relate to a better basic and fundamental understanding at the molecular level of both the various Bt protein toxins, especially those isolated in Egypt, and the insect receptors for the toxins in question. The specific objectives include:

1. isolating toxin genes from subspecies of Bt collected from different Egyptian habitats,
2. sequencing the genes to determine the exact nucleotide sequences of both their regulatory and structural regions,
3. cloning those genes that express the most potent insecticidal proteins into the shuttle vector pHT to study their expression in *Escherichia coli* and the acrystalliferous strain HD-73-26 of Bt,
4. modifying the necleotide sequences of the toxin genes so that maximum expression can be attained in plants,
5. expanding the insect host range of various Egyptian Bt isolates and strains, and
6. identifying, cloning, sequencing and characterizing the insect midgut receptors for toxins of Bt that kill moths and beetles.

The research reported herein is in the form of manuscripts submitted to scientific journals, individual research updates, poster papers for presentation at Pioneer and patent updates. Areas covered in this report include:

- characterization of the natural BT-R_1,
- ligand specificity and affinity of BT-R_1,
- expression of the soluble receptor (BTRE),
- Southern blot analysis of BT-R_1,
- PCR screening for BT-R_1 polymorphisms,
- developmental expression of BT-R_1 in *Manduca sexta*,
- localization of the BT-R_1 binding site for Cry1Ab toxin,
- update on U.S. patent application for receptor for a *B. thuringiensis* toxin, and
- U.S. patent application for *B. thuringiensis* isolates with broad spectrum activity.

Research Achievements

The research to date has produced interesting scientific information on the BT-R_1 receptor for *B. thuringiensis* toxins and on the Egyptian isolate C-18 that has multiple insecticidal activity. BT-R_1 has been further characterized since it was originally cloned and sequenced, and now has been expressed in heterologous cell cultures. BT-R_1 does not co-purify with enzymes such as aminopeptidase, alkaline phosphatase and other enzymes, strongly suggesting that BT-R_1 indeed is the high affinity receptor for Cry1Ab toxins. Work now is in progress to determine the *in vitro* parameters that affect its physical and biochemical stability. Also, attempts are being made to crosslink BT-R_1 to other proteins in the brushborder membrane

and to determine whether BT-R_1 associates naturally with other membrane proteins such as catenins. Preliminary results indicate that BT-R_1 crosslinks to produce higher molecular weight aggregates than the purified natural protein.

BT-R_1 has been expressed in both mammalian and insect cell cultures and the ligand specificity and affinity of the recombinant protein for Cry1A toxins have been determined. A soluble form of BT-R_1 has been expressed in heterologous cell cultures. The receptor is released into the culture medium and, consequently, provides an efficient means of producing large quantities of BT-R_1 for a comprehensive and complete characterization.

Southern blot analysis using various restriction fragments prepared from the cDNA encoding BT-R_1 reveals that there are two discreet regions within the *M. sexta* genome which encode sequences homologous to BT-R_1. However, only one of these genes apparently encodes BT-R_1 whereas the other one is a closely related homologue. PCR screening for BT-R_1 polymorphisms in those genes encoding BT-R_1 has revealed at least four different genotypes with respect to BT-R_1-related sequences. Comparison of the sequences of these proteins should allow us to determine which regions of BT-R_1 are essential in binding toxin as well as mediating toxicity.

Preliminary examination of the expression of BT-R_1 shows that it is highly regulated. Developing *M. sexta* larvae exhibit a significant increase in their level of BT-R_1 mRNA and protein expression as they mature. Furthermore, BT-R_1 expression is highly localized to the midgut of *M. sexta* larvae. Presumably, BT-R_1 expression is regulated by a unique promoter. Isolation and characterization of the promoter region of the BT-R1 gene is being initiated.

Research to determine the Cry1Ab toxin binding site in BT-R_1 has shown that it lies in the last 234 C-terminal amino acids. Apparently, the C-terminal end of BT-R_1 is the location of the Cry1Ab binding site. Additional truncation mutants of a *BamhI-SacI* fragment containing the binding site will be constructed to determine the smallest portion of BT-R_1 necessary for binding, and point mutations will be prepared in the fragment to determine which amino acids are responsible for specific binding of Cry1Ab.

The patent entitled, "Receptor for a *Bacillus thuringiensis* Toxin," apparently is close to issuance. The examiner has indicated that all claims in the application are allowable and has closed prosecution. Once issued, this patent will provide extremely broad coverage for an insect receptor for toxins of *B. thuringiensis*.

A U.S. provisional patent application has been filed for *B. thuringiensis* isolates with broad spectrum activity. Research is in progress to characterize all toxin genes present in an Egyptian isolate of *B. thuringiensis* that has multiple toxicity for lepidopteran, coleopteran and

dipteran insects as well as to at least one root knot nematode. Toxin crystals of the isolate, called C-18, are being purified and the toxic protein responsible for killing corn rootworms is being purified for ligand and binding studies. As soon as a receptor(s) is identified, it will be extensively characterized.

Whitefly Biotypes and Biotype-Specific Transmission of Geminiviruses - Egypt

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Introduction

The economic importance of the whiteflies on Egyptian agriculture has been recognized a long time, but viruliferous whiteflies have become a considerable problem in recent years. The largest and most economically significant group of plant viruses transmitted by whiteflies are the geminiviruses. The tomato yellow leaf curl virus (TYLCV), a geminivirus transmitted by whitefly, has been recorded in most eastern Mediterranean countries. In Egypt, TYLCV causes about 65% yield losses in tomato (FAO report, 1995). For many years, bioassays have been the only tool available to describe the characteristics of viral transmission by the vectors. With the development of polymerase chain reaction (PCR) and molecular hybridization techniques, it is now possible to detect viral molecules in plants and in individual insects. Knowledge of the mode of transmission of viruses from plant to plant by insect vectors is essential for understanding viral epidemiology and for the development of pest management strategies.

Project Goals

1. Identification of different populations of viruliferous whitefly species in tomato using conventional taxonomical methods.
2. Identification of viruliferous whitefly biotypes using protein polymorphism and molecular markers.
3. To study the behavioral aspects and efficiency of geminiviruses transmission by different whitefly species and their biotypes.

Technology and Information Exchange

The U.S. team has provided expertise and protocols for:

- molecular markers for whitefly vectors and geminivirus pathogens,
- exchange of PCR primers sequences and protocols for detection of protein and DNA polymorphism in whiteflies,
- PCR primers for virus detection and DNA sequences of some viruses, and
- detection and identification of geminivirus in the whitefly vectors.

Research Progress

In Egypt, 20 species of whiteflies have been recorded. Two of them, *B. tabaci* and *B. argentifolii,* are considered very important pests for their ability to transmit geminiviruses. During the present study, when we started collecting whiteflies to maintain a mother colony, the heavy infestation of the castor bean whitefly (CBW) *Trialeurodes ricini* (Misra) all over Egypt, drew our attention. This species is recorded here for

the first time in Egypt. The identification of the samples was kindly provided by Dr. Raymond J. Gill, California Department of Food and Agriculture, Sacramento, California, USA, based primarily on pupal cases (Fig. 1). We also used a very clear and distinctive characteristic to distinguish between *B. tabaci* and *T. ricini*—the compound eye (Fig. 2).

A heavy infestation with *T. ricini* was recognized in tomato fields at the experimental station of AGERI. This infestation was associated with a high percentage of tomato plants showing TYLCV typical symptoms (84.8±8.79%). It was of interest to determine whether or not castor bean whitefly is able to transmit TYLCV. It was surprising enough that CBW gave positive results at experimental and molecular levels. In this study, we report for the first time that *T. ricini* is the third whitefly species transmitting geminiviruses.

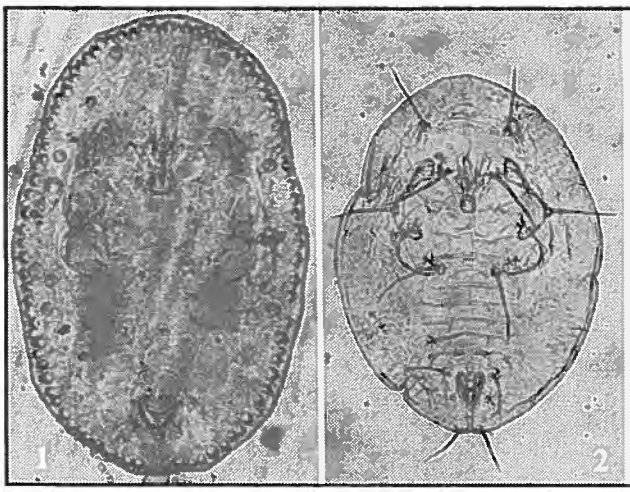

Fig. 1. Pupal cases of *T. ricini* (1) and *B. tabaci* (2).

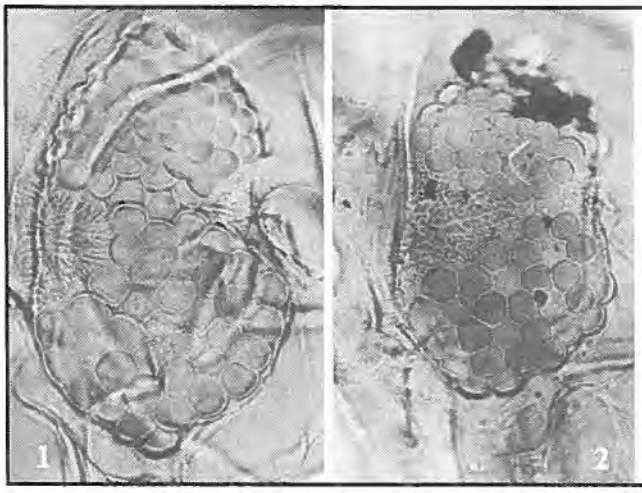

Fig. 2. Compound eyes of *T. ricini* (1) and *B. tabaci* (2), the upper and lower compound eyes of *B. tabaci* are connected by one ommatidium, while they are connected by three ommatidia in *T. ricini*.

The efficiency of TYLCV transmission by the CBW using bioassays was examined. To prove bioassay data, several approaches, at the molecular level have been used in an attempt to detect the virus in the subject whitefly. One of these approaches was enzyme linked immunosorbent assay (ELISA). The double antibody sandwich (DAS) indirect

ELISA data detected TYLCV in the adults of *T. ricini* that were allowed access on infected TYLCV tomato plants. ELISA data strongly supports the bioassay results. PCR, as another approach, was also used to detect the virus in *T. ricini* extracts. Figure 3 shows the specific PCR products which were obtained from extracts of individual *T. ricini* females that had been allowed to access the virus from infected tomato plants. Different specific primer sets were used to amplify the coat protein, the replicase, the intergenic region and the core conserved codons in the coat protein gene of the old world whitefly transmitted geminiviruses. These primers were tested with the purified pTYNA 101 full length viral clone as a positive control. Females of the CBW, which were fed on castor bean for several generations, had no PCR products as was expected.

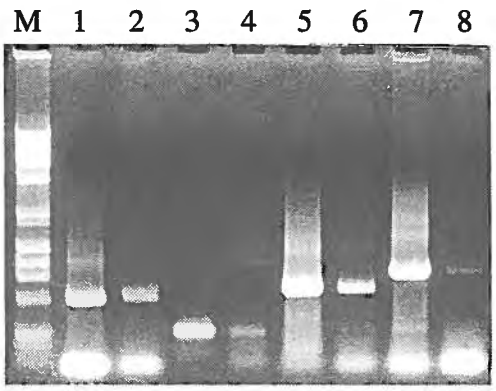

Fig. 3. Polymerase Chain Reaction (PCR) amplification of purified plasmid cloned pTYNA 101 as a positive control and of DNA isolated from individual *T. ricini* adults allowed an access on tomato plants infected with TYLCV-Eg. Sets of primers were used to amplify old world whitefly transmitted geminivirus. OWTG 391-1049 (1, 2), the intergenic region, IR (3, 4), the coat protein, CP (5, 6) and the replicase, C1 (7, 8) whereas M is a marker, the odd numbers represent the purified plasmid cloned pTYNA 101 and even numbers represent DNA isolated from *T. ricini* adults.

Electrophoretic results of adult whitefly total proteins proved that the molecular weight of separated specific protein bands of either *T. ricini* or *B. tabaci* similarly ranged from ~ 14,000 to more than 205,000 daltons. The results emphasized the existence of variation between *B. tabaci* (collected from Egypt and Arizona, USA) and *T. ricini* not only in the number of protein bands but also in the concentration.

The ability to distinguish the biotypes of this newly introduced pest is critical for understanding the biological parameters and population dynamics of this insect. It is also important to determine the economic threshold of this whitefly at which strategic control efforts could be directed. Samples of *T. ricini* adults were

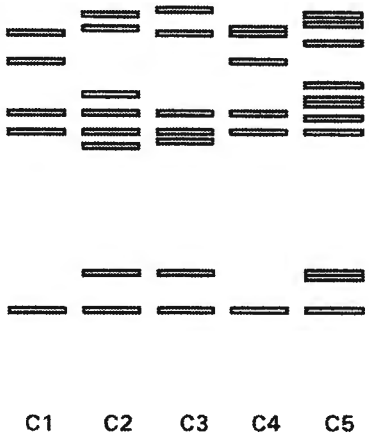

Fig. 4. Esterase patterns of individual T. ricini different biotypes.

Table 1.

Distribution of biotypes of *T. ricini* in Egypt based on non-specific esterase.

Governorte	Locations*	Biotypes	Acquired WF** Geminiviruses
Alexanderia	Alexanderia	C_1	-
Elbohera	Kafr-Eldauwar	C_1	-
	Damanhur	C_5	+
	Ityai Elbarud	C_3	+
Kafr Elsheikh	Kafr Elsheikh	C_4	+
Elkarbia	Qutur	C_1	-
	Karf Elzaiyat	C_1	-
	Tanta	C_1	-
Damiette	Damiette	C_1	-
Eldakahlia	Shirbin	C_2	+
	Elmansura	C_5	+
	Aga	C_5	+
	Mit gamer	C_1	-
Elmenofyia	Quweisna	C_3	+
	Birket Elsab	C_1	-
Elsharkia	Minya Elqamh	C_1	-
	AbuHammad	C_1	-
	Bilbeis	C_1	-
Ismailia	Ismailia	C_3	+
Qalubia	Karf shukr	C_1	-
	Benha	C_1	-
	Tukh	C_1	-
	Qalyub	C_1	-
Cairo	Elmahady	C_1	-
Giza	Giza	C_1	+
	Elaiyat	C_1	-
Elfaiyum	Tamiya	C_1	-
	Sinnurise	C_1	-
	Elfaiyum	C_1	-
Benisuef	Elwasta	C_1	-
	Benisuef	C_1	-
	Biba	C_1	-
Elminya	Maghagha	C_1	-
	Elminya	C_1	-
	Mallawi	C_1	-
Asyut	Manfalut	C_2	+
	Asyut	C_1	-
	Tima	C_1	-
Sohag	Sohag	C_1	-
	Girga	C_1	-
Qena	Nag hammadi	C_1	-
	Qena	C_1	-
	Luxor	C_1	-
Aswan	Isna	C_1	-
	Aswan	C_1	-

*Whiteflies were collected from wild castor bean.

**PCR was used to detect TYLCV in whiteflies.

collected from 45 locations belonging to 19 governorates representing lower and upper Egypt. All these samples of whitefly were collected from castor bean to ignore the host plant interference with esterase patterns.

Figure 4 summarizes and illustrates the esterase pattern differences which have been observed during the biogeographic survey of *T. ricini* populations. Five biotypes of the castor bean whitefly (C_1, C_2, C_3, C_4 and C_5), which are morphologically indistinguishable, have been differentiated based on esterase patterns.

As shown in Table 1, PCR revealed that all C_1 type collections were virus free except the mother colony at Giza which was reared on castor bean and TYLCV infected tomato; however, the other castor bean whitefly biotypes acquired geminiviruses. These results proved the existence of host correlated variation phenomenon in the isozyme patterns.

The acquisition-access period required for the transmitting TYLCV by the castor bean whitefly was detected using PCR. 20±4.68% of *T. ricini* adults acquired TYLCV after 7 hrs of caging with TYLCV infected tomato plants. The rate increased as the acquisition-access period was lengthened and reached 50±7.05% after 10 hrs.

PCR and bioassays were used to determine the inoculation access period. As a fast and reliable tool, PCR was used to detect TYLCV in leaves of tomato plants exposed to viruliferous *T. ricini* for different periods. The TYLCV was detected in 10±2.89% of tomato leaves after 30 min inoculation-access period. The rate increased to 60±8.67% after an hour and reached 90±5.78% after 2 hrs. Similar data were obtained depending on the typical TYLCV symptoms bioassays. The rate of transmission increased as the inoculation access period was lengthened and reached the maximum after 4 hrs inoculation-access period (94±3.46%).

Future Work

1. Comparative morphological studies on mature and immature stages of Egyptian viruliferous whiteflies:
   a. Cotton whitefly *Bemisia tabaci.*
   b. Silver leaf whitefly *Bemisia argentifolii.*
   c. Castor bean whitefly *Trialeurodes ricini.*
2. Detection of esterase electrophoresis. Differences between *B. tabaci*, *B. argentifolii* and *T. ricini,* as well as polymorphism of populations of the same species based on esterase, variability will be studied.
3. Biological and host preference of the Egyptian viruliferous whiteflies.
4. Transmission efficiencies of these selected whiteflies and TYLCV isolates in Egypt.

Micropropagation of Tropical Crops for Commercial Planting - Costa Rica/U.S.

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Agribiotecnología de Costa Rica

Neal Gutterson
DNA Plant Technology

Objectives

The collaborative project between Agribiotecnología de Costa Rica S. A. (ACR) and DNA Plant Technology (DNAP) was initiated with the general goal of implementing more efficient methods of micropropagation for tropical crops, using bioreactor vessels. The project was set up with two short-term crops (banana and pineapple) and two long-term crops (coffee and peach palm).

The short-term objective was to develop and/or adapt liquid culture technology for banana and pineapple. ACR had the responsibility to provide the germplasm of all the target crops. DNAP had the mission to develop the micropropagation technologies and then transfer to ACR the processes and material for the field trial.

The long-term objectives were to implement the production of somatic embryos via bioreactors and to evaluate nursery and fidelity plots micropropagated by the different methodologies.

Research Achievements at DNAP

Funding for the collaboration with Agribiotecnología de Costa Rica (ACR) ended in April 1995.

Banana

During the last few months of the project, the remaining objective for DNAP was to develop a reliable embryogenesis-based regeneration method for the banana cultivar Grand Nain. Using shoots derived from male flowers, we were able to produce axenic embryogenic tissue from a proprietary Grand Nain selection provided to DNAP by ACR. During the funding period we were unable to produce any regenerated whole plants from this embryogenic tissue. A number of approaches have been continued to achieve this, and the cultures themselves (and methods for producing them efficiently) are being provided to ACR.

As reported earlier, DNAP has developed embryogenic tissues from the banana cv. Grand Nain. These tissues have been proliferated both on solid medium and in liquid suspensions. Although both types of cultures proliferate well, we have not been able to regenerate plants from either type of culture. There are two sources for this problem. First, there is a bacterial contaminant present in our cultures. Second, the cultures appear to be in a developmental state that is not suitable for embryo germination. Work in progress is focused on solving these problems.

Axenic Cultures

We have taken two approaches to producing axenic embryogenic cultures. First, we have developed methods to culture the contaminant bacterium in order to characterize it sufficiently so that we might be able to eliminate it through

antibiotic or other treatments. We have found that the bacterium grows preferentially on our standard plant medium conditioned by the growth of bacterial cells. We have also shown that addition of banana extract powder (from Sigma) to the plant medium can support growth of the bacterium to produce turbid suspensions. Nonetheless, the doubling time for bacterial growth is more than two days, even under the best conditions.

For bacterial identification we were unable to use standard methods relying on growth and metabolism of a range of nutrients, since the isolated bacterium grew poorly and only on particular media. Instead, we attempted to identify the organism using a method based on the lipid content of the cell surface. The growth rate is not a factor in this approach. However, the lipid profile obtained was very different from any other bacterium which had been previously analyzed. Thus, we were not able to identify the contaminant in our cultures. Perhaps because the contaminant grows so slowly, we have not been able to eliminate all bacterial cells from any banana cell culture so far.

Our only other approach to producing axenic cultures is to start with fully axenic explant tissue. We have never been able to produce axenic embryogenic tissue from shoot cultures derived from field corms. However, Escalant and his colleagues have been able to regenerate large numbers of plants from embryogenic tissue derived from male flowers. We surmise that one key to his success was the use of male flowers, which, given their position and developmental state, are likely to be axenic. With this in mind, Agribotecnología de Costa Rica has provided us with approximately 100 male inflorescences. We are attempting to produce both embryogenic tissue and shoots from these inflorescences, using immature male flowers as the explant for induction of embryogenic tissue and using the meristem and peduncle tissue for induction of shoots.

Improved Embryogenic Cultures

Modifications in hormones and hormone levels were tested to improve both embryogenic cultures on solid medium and in liquid. A range of picloram concentrations was tested, as well as low concentrations of dicamba (in the range of 1 mg/l), for the effect on culture growth rates and on developmental stage. At low concentrations of picloram (0.1 to 1 mg/l), growth rate was reduced and the embryogenic tissue became white rather than the creamy-yellow color of tissue proliferated at a picloram concentration of 5 mg/l (standard conditions). The enlarged structures observed at low picloram concentrations were not better able to regenerate than the smaller structures observed at the higher picloram concentrations.

Suspension cultures have been reported as the best source of embryogenic tissue for plantlet

regeneration. Our typical suspension cultures of Grand Nain are not as finely divided nor as friable as those reported to give the best regeneration. At the beginning of this reporting period, a new tissue type was observed in suspension culture. It was much more like that reported by Novack and his colleagues. This tissue was separated and cultured independently. These cultures grew with a doubling time of between one week and two weeks for over a month but the doubling time gradually increased to over one month. Initial experiments to germinate plantlets using this suspension have not been successful.

Our current plan is to produce axenic embryogenic tissue from the inflorescence tissue, and then to repeat a number of proliferation and regeneration experiments with the presumably axenic material.

Summary of Status

We continue to collaborate with ACR on the development of an embryogenic method for micropropagation. Other methods tested for advanced micropropagation of bananas, in particular the bioreactor-based methods, were not useful.

Research Achievements at ACR

Pineapple

The pineapple micropropagation methodologies using bioreactor vessels in permanent and temporary immersion, using both large and small containers (PIFU's = filter units) were transferred to ACR by DNAP.

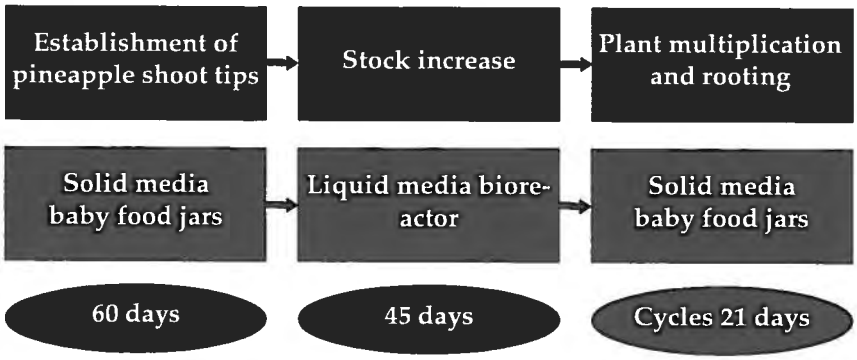
During the period 1994-1995, we particularly concentrated on the implementation and evaluation of PIFU's in combination with an alternative approach for shoot multiplication that consisted of splicing the whole crown 2-3 times (leaf base regeneration, LBR) or sections of leaves (longitudinal leaf regeneration, LLR). In both cases explants came from pineapple shoots established previously *in vitro* and obtained from fruit crowns.

Applying these alternative methods have proven to be very efficient since it takes less time for the shoots to regenerate if compared with the cultures started with whole shoot tips which usually takes 4 months. Additionally, from one single established shoot, it is possible to obtain several explants for inoculation in PIFU's. Maintaining a high degree of sanitation is an important aspect of this system. Results from experiments with clones OA-293 and OA-294 showed a rate of multiplication of 1:24 for LBR, 1:12 for LLR and 1:5 for the controls. These experiments presented clear evidence of the superiority of the LBR method over the traditional system of micropropagation.

The impossibility of clump subdivision presented with all the bioreactor systems was a permanent inconvenience to obtaining large numbers of uniform plants ready to be

transplanted for hardening in the greenhouse. In order to solve this problem, and to take advantage of the efficiency of micropropagation using the PIFU's, we developed the following strategy for pineapple micropropagation.

Chart 1



As we can see in Chart 1 above, the use of filter holders (PIFU´s) for the micropropagation of pineapple in liquid media has proven to be efficient; it can be a useful tool in the first steps of micropropagation and also for keeping clean material for stock purposes.

During 1995-1996, most of the efforts on pineapple were concentrated on the field evaluations on plant material obtained by the different methods of micropropagation mentioned above. Most of this material was produced by DNAP and sent to us in stage III. The main objective of the field evaluations has been to test the clonal fidelity of the pineapple plants. It is well known that pineapple plants present a high level of genetic instability when propagated *in vitro*. It is not rare to find spines on the leaves and different grades of variegation, and with less frequency we can find fruit deformations like collar, which is the generation of slips from the base of the fruits, bottle neck, which is a deformation in the upper part of the fruit; other abnormalities were found in very low percentages.

Based on the classification of plants received from DNAP (groups I, II, III, IV, V), which includes plants derived from somatic embryogenesis, leaf regeneration and bioreactors showed less than 5% abnormal fruits and less than 5% mortality at the field level. These results are good and it is evident that for pineapple the screening done at the greenhouse and the nursery stage before the transfer to the field is efficient since at these levels the percentage of mutation found were of 30-45% for bioreactor and leaf regeneration derived plants and up to 45-64% for the plants derived from somatic embryogenesis.

At the field level all the plants showed good agronomic performance; the bioreactor derived plants had an average height of 50-60 cm and

weight around 2 Kg. The plants derived from somatic embryogenesis were less developed and showed a disparity in growth which ranges between 25-50 cm.

In conclusion, the information presented above shows that pineapple presented a higher rate of mutation when multiplied in bioreactors, due probably to a combination of factors including the liquid, the agitation, the source of explants, and the method of regeneration (leaf regeneration and somatic embryogenesis). Nevertheless, these systems represent a good possibility to increase the number of plants in a short period of time. To be able to use this technology in a commercial production of pineapple, more research is necessary on the identification, characterization and selection of stable lines.

Banana

Banana shoot multiplication has been accomplished in liquid cultures using erlenmeyers, flasks, bioreactor vessels and filter unit systems (PIFU's).

The bioreactor technology still has several limitations because banana tissue in general does not perform well in permanent immersion bioreactors (5 l, 10 l) if compared with the traditional system using solid media and baby food jars. With the temporary immersion system ACR was able to successfully propagate banana and plantains in PIFU's and bioreactors.

There are two main limitations with the bioreactor technology which need to be solved. The first is a contamination caused by an endogenous bacteria frequently present in tissues that grows fast in liquid media. We found that after inoculating a bioreactor with a population of explants previously established *in vitro* and apparently clean, frequently the contamination appeared after 2-3 weeks. This contamination problem was also reported by DNAP as a serious limitation during callus induction experiments.

The second limitation, found during the development of the bioreactor technology for banana micropropagation, was the impossibility of clump subdivision during the process. Normally one banana explant inoculated in the bioreactor generates 2-5 new shoots in a very compact clump. In this situation there are one or two dominant shoots, and an apical dominance system is established; consequently, a reduced number of well developed plants are obtained according with the quality standards used at ACR. As a result of this apical dominance, an important number of small size plants, in different stages of development, and mostly without roots, is generated. Ideally those plants need to be separated and re-inoculated in other bioreactor or in solid media to finish stage III. This alternative includes a high risk of contamination in addition to extra cost in labor, media, shelf room, etc. Because of the limitations indicated above, efforts

were concentrated on the search of a different explant other than the traditional shoot tip. More than 5,000 banana young male flowers were inoculated in order to induce callus. This alternative source of explant was mentioned in a paper published by J. V. Escalant (1 994) as good for callus induction and somatic embryogenesis in banana. Nevertheless, neither DNAP nor ACR were able to efficiently repeat Escalant's protocols. However, during our different experiments with young male flowers, the explants treated with different growth regulators presented organogenesis and new shoots were obtained from these explants. The important issue here is that we were able to obtain shoots free from the cryptic bacteria; 35,000 plants produced by this methodology were planted in the field for clonal fidelity tests and the results were less than 4% off-type plants.

The main objective this year on banana research has been the initiation of embryogenic cells--it still is difficult and time consuming. Different efforts have been made at DNAP and ACR with different sources of explants and different media. Even though at first the results were not encouraging, lately things have changed and we expect better results soon.

Finally, it is important to recognize that a considerable advance in the study of new alternative methods for banana propagation were made. Some were small steps whose impact is difficult to evaluate, and others were more significant, but it is important to recognize the importance for the improvement of banana propagation methods knowing that traditional methods are very inefficient. Also, it is important to remember that bananas imported from the Americas, Caribbean, Africa, the Philippines and elsewhere consist almost entirely of fruits from cultivars of the "Cavendish subgroup (AAA)." Cavendish represents only about 13% of the global production of bananas. The remaining 87% includes a wide diversity of varieties grown by millions of small holders, mainly for local consumption and regional trade in highly-diversified eco-regions around the world. So, any improvement in plant propagation methodologies that can reduce the cost will have a tremendous potential and socioeconomic impact because it opens the opportunity to public and private organizations and growers to rapidly access new genotypes of bananas, with new characteristics like those liberated recently by FHIA with resistance to Black Sigatoka. Any advance on banana propagation will open a new opportunity for a more sustainable production for small and big growers in marginal areas around the world.

Coffee

In coffee, two commercial short-stature varieties were selected for this project: Red Catuai and Catimor OA - 191. The first variety was provided by DNAP and the second by ACR. At the beginning of this project, DNAP had

suspensions of Red Catuai and were called the "old CTR cell lines."

Concerning technology transfer, some of the objectives of this project dealt with the induction and isolation of friable embryogenic tissues (FET), the establishment of FET cell cultures, multiplication, germination and plantlet production. The process of this technology transfer was based on the "old CTR cell lines" which demonstrated the ability to produce a large number of single embryos after 36 months of continuous culture at DNAP.

These cultures were used as a model to train personnel on the induction and production of somatic embryogenesis derived plants, and to increase plant stocks for field evaluation.

The somatic embryogenesis derived plants received from DNAP on March 28,1994, were transferred to the field on June 2, 1995. At the moment we have 364 plants that came from 49 lines. These plants were planted with the objective of testing clonal fidelity. It is interesting to remember that these lines were kept *in vitro* for more than 8 years. Since coffee is a perennial plant we will require at least 3 more years to get to some conclusions concerning the agronomic potential for large-scale production of these coffee plants.

The technology transfer protocols for the micropropagation of coffee using somatic embryogenesis were delivered by Maro Sondahl.

Palm

The method of somatic embryogenesis on palms using sexual embryos as explants was developed by DNAP and transferred to ACR.

Several hundred peach palms were produced. The main problem with the implementation of the methodology was the hardening process. A literature review on the subject showed that many other palms have shown the same difficulty when grown *in vitro*.

This methodology is only for sexual embryos, is expensive and long-term, and has only limited application. For these reasons and because funding has been reduced, a decision was made in 1993 to discontinue research activities in this area.

Constraints

1. The initial project time line, funding, and technical difficulties may have been overly optimistic. In addition, moving technologies developed and reported in research laboratories into commercialization is a process with many potential challenges.
2. Changes in DNAP (ownership, location, consolidation, staffing) caused delays in the project and constraints in the project outcomes. Dr. Maro Sondahl was the key researcher involved in the project at the outset. His departure from DNAP at an early stage of the work resulted in delays and

restarts and may have reduced the project's overall accomplishments of the project. Dr. Sondahl had a relationship with the country project leader that preceded the project establishment, and his leaving not only removed the expertise he directly contributed to the project but also the personal link that is critical to good communication and information flow. DNAP California-based staff were presented with a project that had been conceived by others and was already underway, and they were expected to pick up and move on.

The movement of DNAP from New Jersey to California not only caused a major shift in the U.S. people with the project, but it also moved the project from working with a facility and staff focused mostly on transformation. Changes in ownership most likely presented internal DNAP challenges to keep this project a priority among competing priorities. The physical move resulted in lost and damaged plant material and delays in research.

3. Cuts in the ABSP budget in 1993 further limited project outcomes. Both DNAP and ACR curtailed work on pineapple and banana, and work on ornamental palms and coffee was eliminated.

Indonesia Small Grants Programs

Darmono Taniwiyono
Biotechnology Research Unit for Estate Crops

Use of Immunoassay Probe for Detection and Monitoring of Phytopthora spp., a Causal Agent of Pod Rot of *Theobroma cacao*

Background

Cocoa is an important crop in Indonesia with more than 58% of the 221,000 ton total production coming from smallholdings. The Indonesian government will expand the cocoa plantation from 130,000 ha during the next five years to meet the increasing world demand for cocoa. The existing plant materials in Indonesia (Purwantara, 1990, Purwantara and Pawirosoemardjo, 1990) and other cocoa producing countries are susceptible to pod rot disease caused by Phytophthora spp.

Forty percent of pod loss is usually from damage due to the disease (Purwantara, 1992). Chemical control to protect healthy tissue from new infection has been the main strategy for many years worldwide. However, the use of fungicides is hazardous to man, animals, and beneficial micro-organisms, and lessens the value of associated plant products in the world markets.

It is generally known that fungal propagules in soil are the main source of primary infection, however, disease control toward the reduction of inoculum potential in soil has never been attempted. This is probably due to the lack of techniques to quantify the level of population or amount of inoculum of Phytophtora spp. in soil on cocoa plantations.

Biological preparations for disease diagnosis and pathogen detection are usually highly accurate but too slow and amenable to large scale application (Miller and Martin, 1988). Immunoassay and nucleic hybridisation systems have been used for pathogen detection and disease diagnosis. These molecular probes are more specific, rapid and sensitive than those of conventional methods based on disease symptomatology (Leach and White, 1990). However the use of immunology technology offers greater simplicity and needs less equipment than the use of DNA probes.

Serological methods for detection of plant viruses have been available to pathologists for many years (Torrance and Jones, 1981). However, the scope of application of the methods has expanded to the diagnosis of diseases caused by many different viruses, bacteria, spiroplasmas, mycoplasma-like-organisms (MLO), and fungi (Miller and Martin, 1988). At present there are many research groups and individuals utilising immunological techniques for plant pathogenic fungi (Brown, 1993). Several different immunoassays are now available and widely applicable (Miller and Martin, 1988, Causen et al 1991, Tsuda et al, 1992). Immunoassay has the potential to detect and quantify pathogen propagules in the soil and other complex matrices (El-Nashar et al., 1986).

Objectives

1. To investigate if Phytophthora associated with cacao in Indonesia consists of several serotypes.
2. To produce polyclonal antibodies (PcAbs) against Phytophthora spp. association with cocoa pod rot disease.
3. To investigate its potential for assessing inoculum potential of Phytophthora spp. in soil.

Achievements

Four types of antigens to extracellular polysaccharide total protein, fixed mycelium and unfixed mycelium were used in the elicitation of antisera and yolk antibody by injection of Red Island laying hen. Antigen from extracellular polysaccharide and total protein were found to be more immunogenic and rapidly induced antibody production. Using an indirect ELISA test, it was found that anti-extracellular polysaccharide antibody (PcAb a pol. Phytop) was more specific than other antibody preparations. The binding site of PcAb a pol. Phytop has been identified to be present at 36, 25, 23, 20 and 17 kD. PcAb a pol. Phytop developed in the current study was capable of detecting antigenic material in sterile soil inoculated with the pathogen. Research is in progress to test this antibody for detecting fungal propagules in soil and other field materials in cacao plantation. This research progress will be presented at the International Conference of Biotechnology, June 1997, in Jakarta.

Regeneration Study of Indonesian Sweetpotatoes (*Ipomoea batatas* [L.] Lam)

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Background

Problems in sweetpotato (*Ipomoea batatas* [L.] Lam) production in Indonesia are mainly caused by sweetpotato weevil (*Cylas formicarius* Fabr.). It is difficult to use conventional breeding to confer resistance to sweetpotato weevil, but the application of recombinant DNA technology in sweetpotato has been limited mainly due to inability to regenerate plants efficiently.

Objectives

The objective of this research is to study *in vitro* culture ability and regeneration of seven Indonesian sweetpotato cultivars.

Three types of explant (leaf, tuber, and root) of seven Indonesian sweetpotato cultivars (namely Bentoel, Bis 182-81, Bis 183, Borobudur 4, G-22, Keleneng, and SQ 27) were used for the research. *In vitro* mother stock from those cultivars were prepared for leaf and root explants, while tuber explants were obtained fresh from the field. Explants were passed through 3 different media. Basal medium consisted of MSO (Murashige and Skoog, 1962) pH 5.8 containing MS salts and vitamins, 0.5 g/l casein hydrolysate, 30 g/l sucrose, and 2 g/l phytagel as a solidifying agent. In step 1, auxins such as 2,4-D (0, 0.1, 0.5 mg/l) and NAA (0, 0.05 mg/l) and cytokinins such as BAP (0, 0.1, 0.5 mg/l) and Thidiazuron (0, 2.0 mg/l) were added to MSO to induce callus formation. Cultures were incubated in the dark at 25 + 2 °C. In step 2, calli derived from step 1 were subcultured into MSO supplemented with BAP (0, 2.25 mg/l) combined with GA_3 (0, 10.0 mg/l). In step 3, differentiated calli from step 2 were subcultured to MSO to induce plantlet regeneration. All cultures in step 2 and 3 were incubated at 22 + 2 °C under fluorescent light for 24 hours. Plantlets obtained from step 3 were acclimatised using 1/2 strength Yoshida solution and gradually were transferred to plastic pots and placed in the greenhouse.

Achievements

Callus could be induced from leaf explants by all media. Calli induced by thidiazuron were green, while calli induced by 2,4-D and NAA were creamy. From the seven cultivars used in this research, only four regenerated to form complete plantlets. They were Bis 182-81, Bis 183, Borobudur-4, and SQ 27. Bis 183 gave the best response. Calli induced by thidiazuron could not form plantlets but only green spot calli. In tuber disc regeneration studies, calli could be induced by seven different media except for SQ 27 which produced calli only in SP1 medium (Herman et al, 1995). Only three cultivars (namely Bentoel, Bis

183, and Borobudur-4) responded to form complete plantlets. In the root regeneration study, the calli derived from explants were not as good as calli from leaf and tuber explants. Generally, the calli become brown and failed to form plantlets. Only Bentoel, Bis 182-81, and Borobudur-4 complete plantlets.

Ginger Micropropagation Using Bioreactor System

Kariana Safarman
PT Fitotek Unggul

Background

Ginger (*Zingiber officinale* Rose) is a herbaceous plant which is indigenous to Indonesia and is widely spread throughout India and China. Ginger rhizomes are among herbal tuber roots that are used as spices, as basic ingredients in making of herbal medicine, and as raw material in the food, beverage and pharmaceutical industries. Compared to other spice plants, ginger has a high economic value. The demand for fresh and dry ginger as well as its essential oil in the world market is high. This is one opportunity to promote international trade. Domestic market demand of ginger is still very great due to the expansion of traditional medicines (jamu), food and beverage industries. Until now, Indonesia played a minor role in world ginger trade since traditional agriculture of the commodity is practised. This practise is less concerned with high yield than regularity.

Conventionally, ginger is propagated through rhizomes. One hectare planting area needs about 1-3 tons rhizomes. Farmers usually take planting material from their own product. This practice causes problems once the farmers use infected rhizomes. Disease will then accumulate in the same location and gradually decrease productivity. It is reported that preventive treatment to reduce pathogens in rhizomes can increase the yield three times (Hosoki & Sagawa, 1977). To fulfill the demand of continuous supply, farmers should use clear material and practise good farm management. Biotechnology offers the technique of plant micropropagation. This technique has many advantages over other methods. Among other advantages, the most important arethe ability to provide seedlings year round and the possibility to use it as tool in obtaining disease-free plants. Several researchers have conducted and developed micropropagation methods for rapid clonal multiplication of ginger (Bhagyalakshmi & Sing, 1988; Noguchi and Yamakawa, 1988; Wang, 1988; Balachadran et al 1990).

One integrated ginger plantation which would support a small ginger processing unit would require about 500-1000 ha area for planting. It is calculated that each hectare will require 40,000-70,000 seedlings. The need of seedlings, therefore, cannot be fulfilled entirely by using the current micropropagation system that is characterised by solid culture. This "old" system will put the process into a high-cost situation.

Achievement of ginger micropropagation in agitated liquid culture has led to the use of a bioreactor combined with a solid culture system to cut down on production space and facilities. The application of a bioreactor system plays a very important role in increasing the production

capacity, since it boosts multiplication without raising the cost for labor, raw materials, space and maintenance. In the development of bioreactor methods, the former type of constant immersion culture system was altered towards a more dynamic system. This system, the periodic immersion system, allows shoots to immerse in the media for an exact time, alternating with periods where the plantlets are not submerged. Therefore, the system allows every shoot to reach a higher CO_2 level in the air rather than being submerged in a static immersion system. The equipment for the periodic immersion system is more complex in order to support the flushing of media.

Ginger rhizomes var.Gajah were wiped with Atonik liquid to induce sprouting and the allowed to stand in a cool, dry room. Meristems from the sprouts were taken after a thorough sterilisation of explants. These meristems were then put on a paper bridge inside culture vessels containing Murashige & Skoog solid media supplemented with 5.0 mg/liter Benzyl Adenine (BA) and 0.5 mg/liter Naphthalene Acetic Acid (NAA) and 2% sucrose. The pH was adjusted to 5.7 prior to autoclaving at 121°C for 15 minutes. All culture were maintained in culture room (24-25°C) at 60% relative humidity with 12 hour light period. Propagated shoots are then transferred into 250 ml erlenmeyer flasks and maintained on the shaker for one week. This checks for contamination before putting the shoots onto bioreactor vessels. Clean shoots from every three flasks were then transferred into a 10-liter vessel. Harvesting of plantlets was done after 8 weeks of maintenance in the vessel. According to the stages, flushing of media during this 8-week time divided into:

1. *Shoot multiplication stage*

   It took four weeks for this process and two different combinations of media were used:

   a. MA + BA 5.0 mg/liter (two weeks)
      MS + BA 5.0 mg/liter (two weeks)
   b. MS + BA 7.5 mg/liter (two weeks)
      MS + BA 10.0 mg/liter (two weeks)

2. *Shoot elongation and root induction stage*

   This process took four weeks. Media for this stage is one of the same composition as the above stage but without hormones. Two different frequencies of immersion were used to determine the adequate nutrition absorption by the propagation shoots (5 and 10 times of immersion per 24 hours). Plantlets from the rooting stage were then transferred to acclimatisation boxes containing a mixture of sterile sand and compost (1:1). The 60 x 60 cm boxes were covered with perforated transparent plastic to maintain high humidity. Acclimatisation took about 6-8 weeks. All plantlets were transferred to single polybag for the hardening process. For this stage each polybag contained a mixture of chaff, manure and burnt soil (5:1:2). Field planting was carried out 8 weeks after hardening with the density of 40,000 plants per hectare. Harvesting was done

four months after planting. Rhizomes were picked up from the field carefully and washed under tap water to avoid injuries.

Achievements

Meristems placed on the bridge with liquid media gave better response compared to those on solid media because of the ease of nutrient absorbency. The first multiplication stage was aimed to provide enough shoots for starting material for further bioreactor processing. In solid media, the rate of multiplication was 3-4 every four weeks. From the above results it is assumed that a bioreactor culture system could support a higher plantlet multiplication rate compared to the solid culture. The development of the shoots were good; they were green and healthy. The stage of shaking for one week in the erlenmeyer flask was important to prevent contamination and a bigger loss in the latter stage (bioreactor stage).

Plantlets harvested from the bioreactor were divided into three different grades based on their sizes.

In the acclimatisa-tion stage, plantlets were planted in boxes containing a mixture of sterile sand and compost (1:1). Boxes were covered with perforated transparent plastic. Plastic covers were opened gradually depending on humidity conditions. After 6-8 weeks in the acclimatisation stage, all plantlets were transplanted into polybags for hardening. A mixture of chaff, manure and burnt soil (5:1:2) is used as the media in this stage. Passing the hardening stage in polybags took 8 weeks, plantlets were then planted in the field with a planting density of 20.0x40.0cm. The field temperature is 28-32°C during the day with relative humidity 70-80%. After a four-month planting period, the soil was dug carefully in order to avoid injuries and the ginger rhizomes were harvested.

The last period of the bioreactor process is expected to induce the elongation process from the multiplied shoots. Accumulation of Cytokinine, which was during the multiplication stage, should be reduced first since excessive Cytokinine concentration inhibits shoot

	BAP (mg/L)			Yield (plantlets)			
Immersion	2 wk	2 wk	4 wk	A	B	C	Total
				(>4.0cm)	(2.0-4.0cm)	(<2.0cm)	
5 times/24 hr	5.0	5.0	0.0	189	1,503	4,646	6,338
	7.5	10.0	0.0	24	294	5,131	5,449
10 times/24 hr	5.0	5.0	0.0	6	52	500	558
	7.0	10.0	0.0	-	108	2,815	2,923

elongation. Plantlets harvested from the bioreactor were not initially autotrophic, since they grew in high humidity vessels and were not photosynthetically sel- sufficient. Therefore, they had to pass a transitional period before field planting.

Two phases of the transitional period are acclimatisation and hardening. Plantlets from grade A and B were transferred directly into acclimatisation boxes. The survival rate was 74-80%. Plantlets with grade C (<2.0 cm) were maintained in boxes containing KNOP solution to promote growth and were later treated the same as the bigger size (grade A and B). Plants were harvested after four months growing in the field. The height and weight were measured. The average plant height was about 17-40 cm and rhizomes weighed about 12.7-37.5 grams per plant.

The Development of CVPD Free Citrus Seedling from Protoplast Fusion and Embryogenic Callus Culture

Siti Subandiyah

Gadjah Mada University

Background

The method for isolating protoplasts from the callus of Keprok Siem was established using the enzyme formula IV at pH 5.8 with a three-hour incubation period and slow agitation. This method is expanded with protoplast purification discussed in this report. Additional medium from callus regeneration is tried for Keprok Tawangmanu and seedless lime. The addition of BAP at 5 ppm to MTo+ medium is progressing to produce some shoots from the callus of Keprok Tawangmangu. On the other hand, the callus of seedless lime was green after subculture growing for two months on the medium of MTo+ supplemented with 2 ppm kinetin. Two media compositions were used; however, only the callus of cv. Siem shows good development with vigorous shoots. On the other hand, cv. Tawangmangu produced less shoots and seedless lime did not produce any shoots although the callus was green in color. To solve the problem we would like to use liquid medium and replace the additional 2,4-D with picloram. Hopefully, this will allow us to progress in the regeneration of embryogenic calli.

Protoplast Isolation Leading to Protoplast Purification

Five hundred mg of callus of Keprok Siem is gently fine cut, then suspended in 5 ml enzyme solution (formula IV) and incubated for 3 hours with gentle agitation (70 rpm) at 24 °C. Post incubation, the suspension is filtered using nylon membrane (45 mm pore diameter) and the filtrate is collected in a centrifuge tube. The filtrate is centrifuged at 750 rpm for 7 minutes and the pellet is collected. The pellet is resuspended gently with 5 ml of 25% sucrose in CPW solution. Two ml of 13% manitol in CPW solution is added carefully to top off the protoplast suspension followed by centrifugation at 750 rpm for 7 minutes. The viable protoplasts are at the interface of the two solution layers. The protoplast sediment is collected using a sterile pasture pipette.

Callus Regeneration

As the addition of 0.5 ppm GA is quite good for callus regeneration of Keprok Tawangmangu, some of the callus is subcultured on the medium of MTo+ with 0.1 ppm of 2,4-D, and 1 ppm, 3

ppm or 5 ppm of BAP is added. The callus medium of seedless lime is MTo+ (with 0.1 ppm of 2,4-D) supplemented with 2 ppm of kinetin.

The composition of the media used for callus regeneration is:

1. MTo + 0.1 ppm 2,4-D + 1 ppm BAP
2. MTo + 0.1 ppm 2,4-D + 3 ppm BAP
3. MTo + 0.1 ppm 2,4-D + 5 ppm BAP
4. MTo + 0.1 ppm 2,4-D + 7 ppm BAP
5. MTo + 0.1 ppm 2,4-D + 5 ppm BAP + 0.5 ppm GA
6. MTo + 3 ppm 2,4-D + 2 ppm Kinetin

The medium was solidified with 10 g agar/ liter.

Achievements

The results of the protoplast purification failed several times without viable protoplast collected. However, some progress has been made and the data are presented in Table 1.

The medium of MTo+ supplemented with 5 ppm BAP is the most suitable for callus regeneration of Keprok Tawangmangu and resulted in the development of tiny shoots. The callus of seedless lime become green after subculturing on the medium of MTo+ supplemented with 2 ppm kinetin. Citrus cv. Siem produced 9 shoots on the medium of MTo + 5 ppm BAP or on the medium of MTo + ppm Kinetin. Citrus cv. Tawangmangu produced 15 shoots on the medium of MTo + 0.1 ppm 2,4-D + 5 ppm BAP. Seedless lime did not produce any shoots on these media, but on the medium of MTo + 2 ppm Kinetin the callus became green in color. At the moment we were monitoring the development of shoot regeneration in liquid medium and the use of picloram instead of 2,4-D.

Table 1.

The results on the protoplast purification.

Replication	Number of viable protoplast*	
	before purification	after purification
1	7.8×10^3	1.6×10^3
2	3.1×10^4	2.0×10^3
3	3.2×10^4	1.6×10^3

*=Enzyme formula IV, 3 hr incubation period.

Indonesia Policy Accomplishments

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Biosafety

Guideline Development

A small committee has been assembled to prepare a draft Biosafety Guidelines. Three experts in each of three research sectors (plants, animal, and microorganisms) have been selected. The writing committee has been approved by the Director General of the Agency for Agricultural Research and Development (AARD) of the Ministry of Agriculture. The draft's title is "Guidelines for Planned Introductions into the Environment of Organisms Genetically Modified by Recombinant DNA Techniques."

In order to develop and improve the draft for biosafety guidelines, CRIFC (through the ABSP project) organized a biosafety workshop. The workshop was held at Safari Garden Hotel, Cisarua, West Java, Indonesia on May 28-29, 1996. Forty-five participants from various public and private sector institutions, such as the Bureau of Justice for Ministry of Agriculture, various Research Institutes under AARD, Directorate General for Fishery, Directorate General for Food and Horticulture Crops, Directorate General for Livestock, Directorate General for Estate Crops, Department of Quarantine, Ministry of Health, Ministry of Research and Technology, Department of Environment, the Indonesian Chambers for Commerce, the Indonesian Institute for Science, Inter-University Studies from Bandung Institute for Technology and University of Gadjah Mada, Committee for Indonesian Germplasms, and three participants from non-government organizations (Konphalindo) attended the workshop. The workshop was inaugurated by Dr. Joko Budianto representing Dr. Faisal Kasryno, the Director General of AARD and Dr. Achmad M. Fagi, the Director of CRIFC.

After the formal opening ceremony on the morning of May 28, 1996, the participants were divided into three groups to discuss and develop the appropriate biosafety guideline for Indonesia. These discussion groups consisted of three research sectors (animal, plant, and microorganisms). In general, the discussions were carried out successfully. A chairperson from each of the groups presented their conclusions and suggestions to the workshop participants.

The workshop concluded that:

1. a writing team under AARD will redraft the two versions of guidelines from AARD and Directorate General of Livestock to become one biosafety guideline for agriculture,
2. biosafety guidelines for agriculture will be proposed for a Decree from the Minister of Agriculture, and
3. to finalize the draft of Biosafety Guidelines, a Biosafety Workshop phase II was planned to be conducted by CRIFC between September-October 1996. On June 29, 1996, an AARD writing team met to discuss a new draft of a biosafety guideline which is the result of the combined versions between AARD and the Directorate General for

Livestock. The new draft is called "Guidelines for Biosafety of Agriculture Biotechnology Product."

The AARD biosafety writing team conducted a series of meetings at Bogor to revise the draft of biosafety guideline. The last draft has been reviewed by Dr. Faisal Kasryno, the DG of AARD and the people in charge in the Bureau of Law at the Ministry of Agriculture.

Biosafety Containment Facility

ARMP II funded the construction of a biosafety containment facility. The site of the containment is close to the Molecular Biology Division building of the Research Institute for Food Crops in Biotechnology. There are two phases to the containment construction. The first phase of construction which includes a head house and three greenhouses was finished in March 1997. The second phase of construction includes another three greenhouses and will begin in the middle of 1997.

Intellectual Property Rights

Following the IPR workshop held in March 25-26, 1993, which was conducted jointly by CRIFC and ABSP, the Director General of AARD appointed several scientists to be the writing team to draft the Plant Variety Protection (PVP) legislation. Twenty-five people from various private and public sectors were invited for the academic group discussion on April 30, 1996. After the academic discussion, the writing team conducted several meetings to revise and finalize the draft. The first draft of PVP consisted of 100 articles divided into 12 chapters:

1. general term
2. scope of PV
3. application for PV
4. inspection/examination for PVP
5. transfer of PVP
6. cancellation of PVP
7. fee and charges
8. management of PVP
9. claim of rights
10. criminal proceedings
11. investigation
12. closing chapter

At the end of May 1996, the first draft for Plant Variety Protection was officially accepted by the Minister of Agriculture and Mr. Bambang Kesowo, the Secretary of Cabinet through Dr. Faisal Kasryno. The explanation of each article of PVP has been prepared by the writing team. ARMP II funded the finalizing of the draft for Plant Variety Protection. The second academic group discussion was held in December 1996. The writing team met several times to improve the draft. The total number of articles was reduced from 100 to 78. The final academic draft is at the Bureau of Law at the Ministry of Agriculture. Interdepartmental discussion will be held at the end of March 1997.

Management and Networking Report: Achievements and Constraints

Personnel and Contractual Issues

Catherine Ives

Michigan State University

At the mid-term ABSP meeting, changes in management structure were discussed. The management structure moved from a research emphasis to a technology transfer focus. This change precipitated the elimination of the Research Director position and led to the identification of In-Country Coordinators. This new structure is illustrated on page 5 of this document. Special thanks goes to these in-country coordinators who have provided various services linking in-country and U.S. collaborations.

The ABSP project has had a number of personnel changes over the past year.

- Dr. John Dodds, Managing Director of ABSP, has reduced his participation in the project. Dr. Catherine Ives joined the project in October 1996, as Co-Managing Director. Dr. Ives was previously at USAID in Washington, DC.
- Ms. Linda Skiba, Management Secretary, also reduced her participation in the project. Ms. Laura Wheeler moved from the Financial Office to the Management Office and accepted many of the responsibilities previously held by Ms. Skiba.
- Ms. Maria Rabi, Networking Secretary, has left the project to accept another position at Michigan State University.
- After a prolonged illness, the project welcomed back Mr. Bruce Bedford, Associate Director of the ABSP project.

With staff illnesses and changes, the ABSP staff continues to go beyond their defined responsibilities to meet project goals and obligations.

Biotechnology is a fluctuating and rapidly changing industry. The ABSP project has not been immune to these uncertainties. A number of research projects with private sector partners underwent restructuring and mergers with consequent changes in research personnel. Participants in these projects should be commended for maintaining the research focus under difficult circumstances. We have established subcontracts with all three developing country institutions (KARI, CRIFC, ACR), and they have begun their in-country research programs.

USAID is currently processing our request for a funded extension to the project through September 1998. This will allow transfer of transgenic materials from the U.S. and completion of host country research programs.

Budgetary uncertainty, both in total amount and in the timing of funding, continues to be a problem for long-term planning efforts.

Intellectual Property Rights (IPR) Activities of the ABSP Project

Karim Maredia
Michigan State University

Internship Programs

Intellectual Property/Patent Internship Program at Stanford University, April 1993

This program was organized by Prof. John Barton of Stanford Law School. The program was held from April 1-30, 1993. The goal of the internship program was to provide hands-on experience to legal and scientific personnel from developing countries in various issues related to intellectual property rights. In addition, the internship program enhanced communication between those involved in the sciences and those who have responsibilities in legal issues concerning biotechnology.

The ABSP project sponsored seven participants from collaborating countries. In addition, one participant from Brazil also attended the program.

Intellectual Property Rights and Technology Transfer Internship Program at Michigan State University, February 1996

A two week internship program in Intellectual Property Rights and Technology Transfer was organized at Michigan State University from February 4-17, 1996. This program was organized by the ABSP project in cooperation with the Office of Intellectual Property and the Institute of International Agriculture at MSU. The goal of the program was to provide hands-on experience to international scientists, administrators and policy makers in the day-to-day handling of intellectual properties within the context of the recent changes in the GATT agreement.

Participants gained insight into the basics of intellectual property management including patents, copyrights, trademarks, trade secrets, plant variety certification, licensing of technologies, patent attorney selection, etc. Participants visited technology transfer offices at MSU, the University of Michigan, MIT, and Harvard University. To foster networking, the participants attended the annual meeting of the Association of University Technology Managers (AUTM), in Charleston, South Carolina.

Workshops

Intellectual Property Rights Workshop, Egypt, January 1994

Through the funding of USAID/Cairo, the ABSP/AGERI Cooperative Project sponsored an intellectual property rights workshop which was held in Egypt on January 24-25, 1994. Over 100 participants from various public and private sector institutions attended this workshop. The goal of the workshop was to create a greater awareness among the Egyptian scientific community in various issues related to intellectual property rights, with special emphasis on intellectual property protection in biotechnology, plant breeders' rights and licensing agreements

The ABSP project published a proceedings of this workshop.

Intellectual Property Rights Workshop, Washington, DC, July 1994

The ABSP project sponsored an intellectual property rights workshop in Washington, DC, from July 11-14, 1994. Forty-four participants representing various USAID programs, other federal agencies, international donor agencies, international agricultural centers, and developing countries attended this workshop. The purpose of the workshop was to present intellectual property rights in biotechnology as an important issue to institutions and individuals involved with international agricultural biotechnology.

International participants included representatives from Brazil, Costa Rica, Egypt, Kenya, Indonesia, Morocco, and Thailand. ABSP sponsored the participation of six representatives from collaborating countries. The ABSP project published a proceedings of this workshop.

Intellectual Property Rights Seminar on the Legal Framework for Technology Transfer, Egypt, September 1995

Under the auspices of the American Embassy in Cairo, the ABSP/AGERI Project assisted in the organization of a two-day seminar on the legal framework for technology transfer from September 4-6, 1995. This seminar focused on the intellectual property rights and technology transfer issues within the context of the recent changes in the GATT Agreement. Over 100 representatives from government and private sector institutions in agriculture and pharmaceutical industry attended the workshop. Several speakers from Egypt as well as the international community served as resource persons.

Patent Application and Examination Workshop, Washington, DC, October 1995

Under the ABSP/Indonesia collaboration, Ir. Robinson Hasoloan Singa and Erni Widhyastari Sinungan from the Ministry of Justice, and Widi Hardjono from the Agency of Agricultural Research and Development in Indonesia, participated in a patent application and examination workshop which emphasized biotechnology inventions. The workshop was organized by the U.S. Patent and Trademark Office, Office of Legislative and International

Participants of the Egypt/ ABSP Intellectual Property Rights Worskhop, Cairo, Egypt, January 1994.

Affairs, Visiting Scholar Program and was held in Washington, DC, from October 23- November 3, 1995, with an additional two-day program emphasizing patent application processing for biotechnology inventions. This training stemmed from discussion during a previous ABSP/CRIFC Technology Transfer Workshop, where it stressed the importance of having patent examination officers participate in this training and also having a representa-tive of the scientific community to act as a resource person for scientists and administrators, and to communicate with the Indonesian Patent Office.

Plant Variety Protection & Patents Workshop, Indonesia, March 1996

The ABSP project, through the support of USAID/Jakarta, organized a two-day workshop on intellectual property rights in agriculture, from March 25-26, 1996. The workshop was organized in collaboration with the Central Institute for Food Crops (CRIFC) in the Ministry of Agriculture. The focus of the workshop was on plant variety protection and patents. Fifty senior representatives from the government and private sector in Indonesia attended this workshop.

The main purpose of this workshop was to assist Indonesia in drafting the new plant variety protection law. Prof. John Barton (Stanford Law School), Dr. Silvia Salazar (Costa Rica), Dr. John Wetherell (Fish & Richardson P.C. in California), and Mr. Richard Wilder (U.S. Patent and Trademark Office in Washington, DC) served as resource persons for the workshop.

Plant Variety Protection & Patents Workshop, Morocco, March 1997

The ABSP project, through support from USAID/Rabat and the Ministry of Agriculture in Morocco, the ABSP project organized a Plant Variety Protection (PVP) Colloquium in Rabat, Morocco, from March 24-25, 1997. The goal of this colloquium was to assist Morocco in the implementation and enforcement of new PVP law currently pending approval from the Moroccan Parliament. Over 250 representatives from governmental agencies and agribusiness community attended this colloquium. Twelve international experts from the United States and Europe served as resource persons. A proceedings will be published shortly.

Consultations

Prof. John Barton, Stanford Law School, has assisted the ABSP project as a special consultant on intellectual property rights issues. He has provided both one-to-one consultations as well as consultation through participating in the ABSP sponsored workshops and internship programs.

Biosafety Activities of the ABSP Project

Karim Maredia
Michigan State University

Internship Programs

Biosafety Internship Program: Guidelines Development, Michigan State University, May-July 1993

The ABSP project organized an eight-week internship program in the U.S. from May 10-July 2, 1993. The goal of this internship program was to assist collaborating countries in the development of biosafety guidelines that would allow them to exchange and test biotechnology products. Seven scientists and regulatory personnel from Egypt, Indonesia, and Kenya attended this program. The interns were exposed to "hands on" applications of biosafety procedures used in safe handling of transgenic plants in laboratories, greenhouses and fields at various public and private sector institutions.

In addition, the interns met and interacted with biosafety experts working at several state and federal agencies such as USDA/APHIS, EPA, FDA, and the Michigan Department of Agriculture. During this program, interns reviewed biosafety guidelines, regulations and permit application procedures used in the United States and other countries. With the experience they gained in various biosafety issues, interns developed a draft set of guidelines relevant to their country's circumstances.

Biosafety Internship Program: Risk Assessment and Risk Management, Michigan State University, August 1996

The ABSP project and the Institute of International Agriculture at Michigan State University, in cooperation with the Information Systems for Biotechnology Program at the Virginia Polytechnic Institute (VPI), organized a two-week internship program in biosafety risk assessment and management from August 11-24, 1996. Fifteen international participants attended this program. The goal of the internship program was to assist developing countries in the field testing of transgenic crops. This program provided participants with a thorough grounding in all aspects of biosafety for environmental release of genetically engineered organisms. It also covered the theory and practice of risk assessment and management in agricultural biotechnology applications. A major component of the program provided practical experience in biosafety evaluation through case studies. The participants visited and reviewed several field trials of the transgenic crops in the Midwestern United States.

Workshops

Latin America & Caribbean Region Biosafety Workshop, Jamaica, May 1993

To strengthen the biosafety regulatory framework in the Latin America and Caribbean region, the ABSP project organized a regional biosafety workshop in Oracabessa, Jamaica, from May 10-13, 1993. The workshop was co-

sponsored by two USAID projects: Agricultural Biotechnology for Sustainable Productivity (ABSP) and the Bean Cowpea CRSP. The Jamaica Agricultural Research Programme (JARP) was instrumental in workshop organization and also acted as the host for forty-two representatives from 12 countries.

The workshop purposes were two-fold: first, to examine the status of biosafety guidelines and regulations in the region for testing and utilization of genetically engineered food crops; and second, to assist participants in developing work plans and recommendations from which to begin building the necessary biosafety policies and guidelines in their respective countries.

Presentations were given by representatives from the public and private sectors from seven Latin American/Caribbean countries, Egypt, Kenya, Indonesia, the International Agricultural Research Centers (CIMMYT, CIAT), UNIDO, and various institutions, companies, agencies and programs from the United States. Regional participants drew upon the breadth of knowledge and experience at the workshop to author recommendations for the development and refinement of biosafety regulatory procedures in their home countries. A proceedings of this workshop was published.

Biosafety Workshop, Egypt, January 1994

To create a greater awareness and strengthen the biosafety regulatory framework in Egypt and the Middle East, the ABSP/AGERI project (through the support of the USAID/Cairo) organized a two-day biosafety workshop from January 26-27, 1994. This workshop involved worldwide experts on biosafety, and scientists and regulatory personnel from Egypt and selected countries in Africa. Over 100 participants from Egypt and selected countries in Africa attended the workshop. Funds to support some regional participants were provided by UNIDO/UNEP. The workshop addressed policy issues, risk assessment and field testing aspects that surround the management and safe use of genetically engineered plants. A proceedings of the workshop was published.

Consultations

Egypt, January 1994

ABSP project personnel, during the ABSP/AGERI project evaluation workshop, provided

Biocontainment greenhouse facility at the Agricultural Genetic Engineering Research Institute (AGERI), Giza, Egypt.

one-to-one consultation to AGERI in the drafting of the biosafety guidelines for Egypt. Dr. Patricia Traynor reviewed the draft guidelines and provided comments.

Indonesia, June 1995

Indonesia has been working to develop national biosafety guidelines. The ABSP project has provided some assistance in the development of these guidelines. To further enhance this process, Dr. Patricia Traynor from the USDA-National Biological Impact Assessment Program worked as a special consultant for the ABSP project, and assisted the biosafety guidelines drafting committee in Indonesia. During this consultation a revised draft was developed for further review.

U.S., November 1996

Dr. Patricia Traynor worked as a biosafety consultant to revise the ABSP Technology Transfer Proposal to address the issues raised by the USAID Biosafety Committee. This proposal was developed to obtain the approval of the USAID to transfer transgenic germplasm to Egypt for greenhouse and field testing.

International Meetings

Bogor, Indonesia, April 1993

ABSP sponsored participation of two experts in a biosafety workshop which was organized by the International Service for the Acquisition of Agric-Biotech Applications (ISAAA) at Cornell University. This workshop was held in Bogor, Indonesia, in April 1993, this workshop was organized to foster regional cooperation in biosafety in Asia. Forty-one participants from five Asian countries attended this workshop.

Harare, Zimbabwe, October 1993

The Special Program for Biotechnology and Development of the Dutch Ministry of Foreign Affairs, sponsored a workshop in Harare, Zimbabwe, from October 11-14, 1993. The workshop was entitled, "Africa Region Conference on International Cooperation on Safety in Biotechnology." Dr. Karim Maredia, from the ABSP project, participated in the conference to serve in working groups and help to develop strategies and mechanisms for regional cooperation and harmonization in the safe use of biotechnology in Africa.

Monterey, California, November 1994

Drs. Madgy Madkour, Karim Maredia, Muhammad Herman, Anggoro Hadi Permadi and Jeff Lowe attended the USDA sponsored third international symposium on "Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms" in Monterey, California from November 13-15, 1994. The workshop provided the ABSP participants with an insight into various issues related to field testing of transgenic

organisms, as well as networking opportunities with biosafety experts worldwide.

San Jose, Costa Rica, March 1995

Dr. John Dodds and Mr. Bruce Bedford participated in a seminar/workshop on "Biodiversity and Harmonization of Biosafety in Central America and Dominican Republic" in San Jose, Costa Rica, from February 28-March 2, 1995. Others with ABSP affiliations who participated were Dr. Oscar Arias and Dr. Ileana Salazar of Agribiotecnologia de Costa Rica (ACR), and Dr. Esther Kahangi, Jomo Kenyatta University in Kenya, who was visiting ACR under support from ABSP. Dr. Salazar presented an overview of the ABSP project and biosafety activities sponsored by the project as one model of efforts to build knowledge and the human resource in regard to biosafety.

Stockholm, Sweden, May 1996

Dr. Karim Maredia and Mr. Bruce Bedford attended an Evaluation Criteria Development Workshop on Biosafety in Stockholm, Sweden, from May 21-23, 1996. The workshop was organized by the Biotechnology Advisory Commission (BAC) of the Stockholm Environment Institute. Dr. Maredia presented a paper entitled, "Capacity Building in Biosafety: Experience of the USAID/ABSP project." The workshop brought together 25 representatives from international donor organizations, developing countries and non-governmental organizations. The goal of the workshop was to articulate evaluation criteria that can be used to plan future activities in biosafety capacity building and measure their effectiveness.

Biosafety Infrastructure Development

Construction of the Biosafety Greenhouse Facility, Cairo, Egypt

The ABSP project assisted AGERI in the construction of the modern biocontainment greenhouse facility in Giza, Egypt. Through the support from the USAID/Cairo, the ABSP/AGERI project, in cooperation with the University of Arizona, the construction of the biosafety greenhouse at AGERI was completed. This greenhouse facility is the first of its kind in the Middle East. It has been inspected and certified by the USDA/APHIS, and Egyptian scientists are now using it for greenhouse testing of transgenic materials.

Sharing of the Egypt/Biosafety Greenhouse Facility Plans with Indonesia

The ABSP project, through an appropriate intellectual property rights agreement, has shared the Egypt biosafety greenhouse facility plans with ABSP collaborators in Indonesia. Indonesian collaborators have reviewed these blueprints and are in the process of constructing their own facility.

Technology Transfer Activities of the ABSP Project

Karim Maredia
Michigan State University

Technology Transfer: Field Tests

In January 1997, the first in-country field test of transgenic material, sponsored by ABSP, was initiated at the Agricultural Genetic Engineering Research Institute (AGERI) in Giza, Egypt. These experiments are detailed in the potato transformation research section of this report.

ABSP/Indonesia Implementation Workshop, Bogor, Indonesia, August 1992

In August 1992, the ABSP project conducted an implementation workshop at the Central Research Institute for Food Crops (CRIFC), in Bogor, Indonesia, to initiate the collaboration between ABSP and Indonesia. The purpose of the workshop was to bring everyone together for firsthand communication and interaction regarding project plans, and to identify candidates for post-doctoral and internship positions based in the U.S. Over 35 scientists, senior administrators and policy makers attended this workshop.

ABSP/Egypt Implementation Workshop, Cairo, Egypt, January 1993

A workshop for the implementation of the ABSP/Egypt cooperative agreement was jointly designed and arranged by the National Agricultural Genetic Engineering Laboratory (NAGEL) Cairo, Egypt, now known as the Agricultural Genetic Engineering Research Institute (AGERI), and the ABSP project at Michigan State University. The workshop was held at AGERI from January 10-15, 1993, and was attended by scientists and government representatives from public and private institutions in Egypt, including USAID/Cairo, the National Agricultural Research Program, the Agricultural Research Centers, AGERI, the Horticulture Research Institute, the Plant Pathology Research Institute, Ain Shams University; and the United States, including ABSP research, management and subcontractor personnel from Michigan State University, Cornell University, Scripps Research Institute, USDA/APHIS, Stanford Law School, and the University of Arizona. Participants focused on developing and reporting first-year workplans based on commodities (cucurbits, maize, potato and tomato) and management areas (biosafety and intellectual property rights, management and network activities).

Egypt Evaluation Workshop, Cairo, Egypt, January 1994

The ABSP/AGERI project evaluation workshop was held at AGERI from January 30-31, 1994, and brought the research, policy, networking, and management teams from AGERI and the ABSP project together to discuss the progress made during the first year of collaboration, and developed detailed workplans on research, networking, policy, and training

activities for the second year of the project.

Indonesia Technology Transfer Workshop, Cisarua, Indonesia, April 1995

The ABSP/Indonesia technology transfer workshop was held in Cisarua, Indonesia from April 18-20, 1995. The purposes of the workshop were to asses the status of environmentally-sound technologies and products developed in the collaboration, and to promote a biosafety and intellectual property rights (IPR) policy environment in Indonesia conducive to their safe and legal transfer. Over 50 representatives from various public and private sector institutions in Indonesia, and ten international participants, attended.

During the workshop, based on the research achievements and the need for further development, workplans were developed and seven areas were identified for funding:

1. Introduction of *Bacillus thuringiensis (Bt)* gene into Indonesian maize germplasm for stem borer resistance.
2. Development of tuber moth and virus resistant potatoes for Indonesia.
3. Development of zucchini yellow mosaic virus (ZYMV) resistant cucumber through genetic engineering for Indonesian agriculture.
4. Improvement of sweetpotato for insect resistance via genetic engineering in Indonesia.
5. The development of seedless watermelon or cucumber through anther and protoplast technology.
6. Strengthening the intellectual property rights (IPR) framework in Indonesia.
7. Drafting biosafety guidelines.

Technology Transfer: Facilitating Commercial Linkages

Visits by Moroccan Scientists to DNA Plant Technology Company, U.S., December 1992

A team of four Moroccan scientists visited Michigan State University, DNA Plant Technology Company, and USAID/Washington, DC, from November 28-December 5, 1992. The goal of this visit was to assess the possibility of building linkages with Michigan State University and DNA Plant Technology with relevance to the Morocco Agribusiness Promotion. Specifically, building collaboration with DNA Plant Technology Company in the area of transformation and cloning technologies for date palm, banana and pineapple.

U.S.-Based Industrial Seminar Series, April 1993

The ABSP project organized a U.S.-based Industrial Seminar Series (ISS) from April 12-19, 1993. The goal of this seminar series was to provide opportunities for senior administrators and private sector personnel from developing countries to interact with technical and business

managers at private U.S. companies which have active agricultural plant biotechnology programs. The seminar series started with participation in the 7th International Biotechnology Meeting and Exhibition of the Association of Biotechnology Companies [now called the Biotechnology Industry Organization, (BIO)], in North Carolina, followed by visits to ICI Seeds in Iowa, Ecogen in Pennsylvania, and DNA Plant Technology in New Jersey. The seminar series was instrumental in opening lines of communication between leaders from host and other countries.

Participation in International Meetings

November 1993, The Hague, The Netherlands

Dr. John Dodds, Dr. Magdy Madkour and Mr. Bruce Bedford participated in the global biotechnology workshop at ISNAR, The Hague, The Netherlands, from November 9-11, 1993. Dr. Madkour presented a paper on the activities of the AGERI/ABSP cooperative project. The AGERI/ABSP project poster was displayed at the meeting. Dr. Dodds provided the keynote address in the session, "Technology Transfer and Building Institutional Linkages." Mr. Bedford acted as reporter for the session, "Communication and Networks: Providing Information Globally," and provided input on the form and utility of the International Biotechnology Program database developed at ISNAR.

May 1994, East Lansing, Michigan, U.S.

The National Agriculture Biotechnology Council held its sixth annual meeting at Michigan State University from May 23-24, 1994. The focus of the meeting was on agricultural biotechnology and the public good. Several ABSP personnel attended this meeting. Dr. John Dodds gave a keynote presentation on, "Agricultural Biotechnology for Sustainable Productivity (ABSP): A USAID Initiative for Plant Biotechnology in the Developing World." Dr. Magdy Madkour, Director of the Agricultural Genetic Engineering Research Institute (AGERI) in Giza, Egypt, gave a keynote presentation on, "Genetic Engineering Addressing Agricultural Development in Egypt: A National Program Perspective." Mr. Bruce Bedford served as a team member who provided recommendations to the NABC on, "Biotechnology and the Environment," and Dr. Karim Maredia served as a team member who provided NABC with recommendations on, "Global Interdependence and Biotechnology."

May 1994, Toronto, Canada

The 8th Annual Meeting of the Biotechnology Industry Organization was held in Toronto, Canada, from May 23-27, 1994. At this meeting, the ABSP project hosted a concurrent session, entitled, "Building Bridges: Promoting Development Cooperation Within USAID's Agricultural Biotechnology for Sustainable

Productivity Project." Dr. Judith Chambers, the ABSP Project Officer chaired the session and the presenters were: Mr. Rustam Effendi, Fitotek Unggul, Indonesia; Dr. Roger Beachy, ILTAB at the Scripps Research Institute, California, U.S.; Prof. Dr. Magdy Madkour, AGERI, Giza, Egypt; Dr. Martin Wilson, ICI Seeds, Inc., Iowa, U.S.

November 1994, Monterey, California, U.S.

Drs. Judith Chambers, Magdy Madkour, Muhammad Herman, Anggoro Hadi Permadi, Jeff Lowe and Karim Maredia participated in the one-day, "Agricultural Biotechnology: Beyond the Laboratory," symposium in Monterey, California, on November 12, 1994. The symposium was sponsored by USAID/Washington, DC, the University of California, the U.S. Department of Agriculture, and the Citizens Network for Foreign Affairs.

May 1995, San Francisco, California, U.S.

The 9th annual meeting of the Biotechnology Industry Organization (BIO) was held in San Francisco, California, from May 20-25, 1995. The ABSP project sponsored a session entitled, "Intellectual Property Rights and Agriculture: An Evolving Global Scenario." The session was chaired by Dr. Judith Chambers, who spoke on, "IPR, Agricultural Biotechnology and Development Assistance." In this session, several prominent speakers gave presentations related to intellectual property rights trends worldwide. The ABSP project sponsored participation of developing country IPR leaders Dr. Norah Olembo (KIPO, Kenya), Dr. Silvia Salazar (University of Costa Rica, Costa Rica), and Dr. Magdy Madkour (AGERI, Egypt).

June 1995, Jakarta, Indonesia

ABSP/USAID co-sponsored the Second Conference on Agricultural Biotechnology (CAB-2) which was held in Jakarta, Indonesia, from June 13-15, 1995. Drs. Patricia Traynor and Karim Maredia participated in this conference. Dr. Maredia gave an overview presentation on the activities of the ABSP project. Following the meeting, Drs. Traynor and Maredia assisted in the revision of the draft biosafety guidelines for Indonesia.

November 1995, Los Angeles, California, U.S.

Drs. Frederic Erbisch and Karim Maredia attended the Pacific Rim University/Industry Technology Transfer Conference which was held in Los Angeles, California, from November 13-15, 1995. This conference was hosted by Michigan State University and Technology Transfer Conferences, Inc. Dr. Erbisch gave a presentation on technology transfer in the U.S. Dr. Maredia gave a presentation on the technology transfer activities of the ABSP project. This unique international conference allowed opportunities for networking, for new technologies, and for new contacts from outstanding Pacific Rim Universities.

February 1996, Charleston, South Carolina, U.S.

Drs. Frederic Erbisch, John Dodds, Judith Chambers, Peter Carlson, Karim Maredia and Mr. Bruce Bedford from the ABSP project attended the annual meeting of the Association of University Technology Managers (AUTM) in Charleston, South Carolina, from February 11-14, 1996. In addition, as a part of the IPR Internship Program, ABSP sponsored the participation of Drs. Achmad Fagi, Ati Duriat and Djoko Damardjati from Indonesia; Dr. Sylvia Salazar from Costa Rica; and Mr. Lucas Sese from Kenya. The AUTM meeting provided networking opportunities for the ABSP group. At AUTM, two special sessions were held, one focusing on "Technology Transfer to Emerging Markets," and the second focusing on "Intellectual Property on the International Scene."

Technology Transfer Framework

The ABSP project, in cooperation with the Office of Intellectual Property at Michigan State, University has developed a standard technology transfer protocol for the exchange of technologies generated within the ABSP project. The ABSP technology transfer framework will include a four-step process as outlined below:

1. Disclosure of technology.
2. Identification of technology in cooperation with ABSP collaborators.
3. Approval for the transfer of technology by USAID, the ABSP project, and appropriate national authorities.
4. Actual transfer of technology.

Networking

Bruce Bedford
Michigan State University

Genetic Transformation Workshop

During May 1994, Dr. Muhammad Herman, researcher at the Research Institute for Food Crop Biotechnology (RIFCB), and Dr. Liswidowati, researcher at the Research and Development Center for Biology (RDCB), both in Bogor, Indonesia, demonstrated transformation techniques to fourteen junior scientists from the Central Research Institute for Food Crops, the Central Research institute for Spice and Medicine Crops, and RDCB.

Drs. Herman and Liswidowati each spent two years as Thomas Jefferson Fellows in laboratories at Michigan State University as an integral part of the ABSP project. Dr. Herman researched systems for sweetpotato regeneration and transformation, and Dr. Liswidowati concentrated her genetic engineering efforts on potato. The May 1994, workshop in Bogor served to further disseminate what the researchers had learned to a broader section of the Indonesian agricultural science community. Training included lectures and demonstrations of various techniques in plant transformation such as how to transfer genes using Agrobacterium using a vortex system, and determining gene expression using Gus assay and ELISA.

Workshop on Conserving Genetic Resources

The June 1994 workshop on conserving and managing genetic resources in Egypt and the Mediterranean region was organized through a partnership of the Agricultural Genetic Engineering Research Institute (AGERI) in Cairo, the ABSP project at Michigan State University, and Genetic Resources Communications Systems, Inc. — publishers of the international news journal DIVERSITY.

The workshop, funded by USAID through Egypt's National Agriculture Research Program (NARP) program, helped to create a greater awareness of the importance of genetic resources in achieving long-term sustainability and food security. It served as a logical follow-up to an eight-day ABSP workshop series on biosafety, intellectual property and biotechnology research held in January 1994, and clearly demonstrated a leadership role for Egypt in fostering regional cooperation in conservation efforts.

Banana Tissue Culture

The banana tissue culture for rapid multiplication is a joint project between Jomo Kenyatta University of Agriculture and Technology (JKUAT) and KARI's National Horticultural Research Program at Thika. The technology is very well developed and the supply

of tissue culture propagated banana seedlings to farmers is at hand. Meeting demands of the increasing number of farmers is the main hurdle. To overcome this problem avenues for commercial scale production are being sought.

Issues of infrastructure and expertise in commercial ventures pose the greatest obstacles and were the main reasons for KARI/ABSP project support for the travel of Dr. Esther Kahangi, JKUAT, to visit DNA Plant Technology in the U.S. and Agribiotecnología de Costa Rica and CATIE in Costa Rica. Dr. Kahangi interacted with tropical crops experts at DNAP and spent time observing the private sector operation at ACR, to formulate ideas on scaling up production of banana seedlings to meet farmer demand in Kenya.

Biotechnology Satellite Symposium

The ABSP project and the Department of Crop and Soil Sciences co-funded the site coordination for MSU scientists in a symposium on "Issues in Biotechnology," sponsored and broadcast by the A*DEC consortium of universities. This symposium was held at Michigan State University during October and November 1996. Approximately 20 scientists attended the satellite symposia on three occasions. Topics investigated were, "Plant Transformation," "Commercialization Strategies," and "Assessing Risk."

IABS Conference

Five Indonesian scientists with administrative support roles for the ABSP project, and those who conducted research under the ABSP/CRIFC small grants program, presented papers at a conference of the Indonesian Agricultural Biotechnology Society during March 1997 in Bogor, Indonesia. These presentations are evidence of one more method of the extended dissemination of ABSP generated information to a broader scientific community through the developing country partners in the project.

Participants

Human Resource Development

Egypt - *Research*			
Dr. Naglaa Abdallah	1991-1992	Scripps Research Institute	Nine-month visit to learn cloning, sequencing and gene construction.
Dr. Nagwa Aref	1992	Scripps Research Institute	Six-month visit to learn cloning, sequencing and gene construction.
Dr. Nagwa Aref	1993	Scripps Research Institute	Three-week visit to perform comparison between serological diagnostic and PCR diagnostic on a set of samples.
Dr. Taymour Nasr El-Din	April 1-25, 1993	Dr. Mariam Sticklen Michigan State University	Isolation and culture of maize immature embryos by using mature embryos. Particle bombardment method of maize transformation. New system of maize shoot multiplication.
Mr. Ahmed Abou-Salha	May 30-July 26, 1993	Dr. Rebecca Grumet Michigan State University	Gene transfer in cucurbits. Learn NPT II ELISA technique, regeneration procedures for melons and microcutting technique.
Dr. M. Soliman Hassasnein Salem	July 30- September 25, 1993	Dr. Mariam Sticklen Michigan State University	Grain experience in the genetic engineering of maize using both shoot multiplication and immature embryo bombardment and culture. "Hands on" experience with techniques related to cellular molecular biology.
Mr. Mohamed Abdel Wahed	August 14, 1993- February 15, 1994	Dr. Claude Fauquet Dr. Roger Beachy Scripps Research Institute	Tomato transformation techniques.
Ms. Nagwa Aref	August 15-29, 1993	Dr. Claude Fauquet Dr. Roger Beachy Scripps Research Institute	Tomato transformation techniques.
Dr. Mahmoud Riffat	August 22- September 11, 1993	Dr. Molly Kyle Dr. Henry Munger Dr. Rosario Provvidenti Cornell University	Become familiar with important strains of ZYMV/CP genes in controlling virus. Learn molecular virology and genetics techniques. Become familiar with breeding procedures involved in cucurbit breeding using lines possessing natural resistance genes, as well as new biotechnological approaches.
Dr. Hanaiya El-Itriby Dr. Ebtissam Hussein	August 24- September 3, 1993	Dr. Mariam Sticklen Michigan State University	Maize transformation techniques. Learn electrophoresis techniques and immature embryo bombardment and culture.
		Dr. Martin Wilson ICI Seeds, Iowa	Interact with ICI scientists to develop new collaboration. Visit to maize transformation research laboratories at ICI Seeds.

Dr. Mohamed El-Wahed	September 1993- March 1994	Scripps Research Institute	Seven-month visit to learn tomato regeneration and transformation, and DNA manipulation.
Dr. Mohamed Saleh	September 7, 1993- March 5, 1994	Dr. Rebecca Grumet Michigan State University	Regeneration and transformation of Egyptian melon germplasm using ELISA test.
Ms. Sireen Assem	September 7, 1993- March 5, 1994	Dr. Mariam Sticklen Michigan State University	Regeneration and transformation of Egyptian maize using particle delivery system.
Ms. Hyam Abdel Kaden	September 7-24, 1993	Dr. Rebecca Grumet Michigan State University	Program discontinued to a family illness.
Mr. Raef Bishara Hanna	September 28, 1993- January 31, 1994	Dr. Jon Sticklen Michigan State University	Computer networking and biocomputing.
Mr. Ismail Abdel-Hamid	October 1- December 18, 1993	Dr. David Douches Michigan State University	Learn potato regeneration and transformation techniques. Learn molecular techniques used to confirm integration of genes in the potato cultivars. Exposure to biosafety issues associated with the harvesting of transgenic potato trials.
Dr. Walid Elyassaki	October 19- December 20, 1993	Dr. Ed Grafius Michigan State University	Learn new techniques in insect pathology and plant engineering including transfer of entomopathic bacterial (B.t.) toxin genes into potatoes, and bioassay techniques for evaluation of resistance.
Mr. Ahmed El Bakry	November 1- December 17, 1993	Dr. Claude Fauquet Dr. Roger Beachy Scripps Research Institute	Regeneration and transformation of tomatoes.
Dr. Naglaa Abdallah	1994	Scripps Research Institute	Five-month visit to produce several clones with different viral genes.
Ms. Shireen Assem	April 7-June 30, 1995	ICI Seeds, Iowa	Maize transformation with B.t. genes.
Egypt - *Industrial Seminar Series*			
Dr. Magdy Madkour	April 18-21, 1993	ICI Seeds, Iowa Ecogen, Pennsylvania DNAP, New Jersey	Participated in the ABSP-sponsored Industrial Seminar Series.

Egypt - *Biosafety*			
Mr. Michael Ghaly Mr. Walid Maaty	May 6-July 7, 1993	ABSP Research and Network Offices Michigan State University USDA/APHIS, EPA, FDA Washington, DC	Eight-week internship program in biosafety including participation in the Latin America/ Caribbean Region Biosafety Workshop in Jamaica.
Dr. Magdy Madkour	August 1-2, 1993	University of Arizona Tucson, Arizona	Discussions regarding detailed plans for the construction of the biosafety greenhouse facility in Egypt.
Mr. Ahmed Wally	August 12-28, 1995	Michigan State University USDA/APHIS, FDA, EPA Virginia Polytechnic Institute USAID Washington, DC	Biosafety internship program for field testing of transgenic plants, and risk assessment and
Dr. Taymour Nasr El-Din Mr. Khaled Essam	August 11-24, 1996	Michigan State University	Biosafety internship program on risk assessment and risk management.
Egypt - *Intellectual Property Rights*			
Mr. Khaled Abdallah Mr. Altef El-Azab Ms. Manal El-Bagdady	April 1-28, 1993	Prof. John Barton Stanford Law School	Four-week internship program in Intellectual Property Rights.
Mr. Atef El-Azab Dr. Taymour Nasr El-Din	July 11-14, 1994	Washington, DC	Participate in the ABSP-sponsored Intellectual Property Rights workshop.
Egypt - *Collaboration/Other*			
Dr. Magdy Madkour	April 13-16, 1993	Research Triangle Park Raleigh, North Carolina	Attended the 7th annual meeting of the Association of Biotechnology Companies.
Dr. Magdy Madkour	July 29-30, 1993	Michigan State University	Interactions with the ABSP researchers and management team in the U.S.
Dr. Magdy Madkour	May 23-27, 1994	Toronto, Canada	Participated in the annual meeting of BIO.
Dr. Magdy Madkour	August 25-26, 1994	Michigan State University	Participated in the ABSP Technical Advisory Group meeting.
Dr. Magdy Madkour	May 20-25, 1995	San Francisco, California	Presented a paper at the BIO annual meeting.
Dr. Magdy Madkour	March 20-28, 1996	Cornell University ICI Seeds, Iowa Scripps Research Institute University of Wyoming University of Arizona	Review the progress made under the ABSP Egypt cooperative project.

Mr. Ahmed Mohamed Ahmed Mr. Gamal Ebrahim Hardy Osman	July 21-August 9, 1996	Michigan State University	Participated in a short course in Integrated Pest Management.
Costa Rica - *Research*			
Dr. Neal Gutterson Dr. Dean Engler Dr. Ebrahim Firoozabady		Agribiotecnologia	Four-day visit to provide technical training on bioreactor technology, and to learn about clonal fidelity tests and problems of micropropagation.
Dr. Ileana Salazar Dr. Jose Antonia Montero		DNAP, California	Five-day visit to learn about bioreactors.
Costa Rica - *Industrial Seminar Series*			
Dr. Oscar Arias	April 17-23, 1993	ICI Seeds, Iowa Ecogen, Pennsylvania DNAP, New Jersey	Speaker at the 7th Annual American Biotechnology International Meeting, and ABSP Industrial Seminar Series.
Costa Rica - *Biosafety*			
Dr. Ileana Salazar	May 10-13, 1993	Jamaica	Participate in the Latin America/Caribbean Region Biosafety Workshop.
Dr. Ileana Salazar	August 11-24, 1996	Michigan State University ICI Seeds, Iowa University of Wisconsin	Biosafety Internship Program: Risk Assessment and Risk Management.
Costa Rica - *Intellectual Property Rights*			
Dr. Silvia Salazar	July 11-14, 1994	Washington, DC	Intellectual Property Rights Workshop.
Dr. Silvia Salazar	February 4-17, 1996	Michigan State University Association of University Technology Managers	Intellectual Property Rights and Technology Transfer Internship Program.
Costa Rica - *Collaboration/Other*			
Dr. Oscar Arias	August 25-26, 1994	Michigan State University	ABSP Technical Advisory Group meeting.
Dr. Oscar Arias	November 9-13, 1994	Monterey, California	Speaker in the plenary session of the Symposium, "Agricultural Biotechnology Beyond the Laboratory."
Dr. Silvia Salazar	May 20-25, 1995	San Francisco, California	Present a paper on IPR at the BIO annual meeting.
Kenya - *Research*			
Mr. Duncan Kirubi	September 15, 1993- September 18, 1996	Dr. Roberta Smith Texas A&M University	Ph.D. degree program in maize transformation with B.t. genes.

Dr. Esther Kahangi	February 1995	DNAP Agribiotecnologia CATIE	To interact with tropical crops at DNAP and observe the private sector operation at ACR, and to formulate ideas on scaling up production of banana seedlings to meet farmer demand in Kenya.
Dr. John Wafula	August 9-18, 1995	Michigan State University Asgrow Seed Company Monsanto Company Cornell University	To interact with ABSP management team and ABSP researchers.
Ms. Charity Macharia	August 14- October 3, 1995	Michigan State University	Collaborative research in genetic engineering; learn techniques for genetic transformation for PVY/PVX virus in potato.
Ms. Charity Macharia	October 4, 1995- June 1, 1996	Monsanto Company St. Louis, Missouri	Learn techniques for gene transformation technologies in fetherly mottle virus including learning micropropagation techniques for genetic transformation of sweet potato in readiness for exportation to Kenya.
Ms. Charity Macharia	June 2-13, 1996	Michigan State University	Learn techniques for genetic transformation technologies in fethery mottle virus including learning micropropagation techniques for genetic transformation of sweet potato in readiness for transfer to Kenya.
Kenya - *Industrial Seminar Series*			
Dr. J. K. Rutto Dr. Florence Wambugu	April 12-19, 1993	North Carolina ICI Seeds, Iowa Ecogen, Pennsylvania DNAP, New Jersey	Industrial Seminar Series.
Kenya - *Biosafety*			
Dr. Glynn Madumandu Mr. Francis Nang'ayo	May 10-July 2, 1993	Michigan State University USDA/APHIS, EPA, FDA	Biosafety Internship Program guidelines development.
Dr. John Wafula Ms. Mary Wabule Mr. Lucas Sese	January 24-31, 1994	Cairo, Egypt	Biosafety and Project Evaluation workshop series.
Ms. Charity Macharia Dr. Chagema John Kedera	August 11-24, 1996	Michigan State University ICI Seeds, Iowa Asgrow, Michigan University of Wisconsin	Biosafety Internship Program: Risk Assessment and Risk Management.

Kenya - *Intellectual Property Rights*			
Dr. John Wafula Ms. Mary Wabule Mr. Lucas Sese	January 24-31, 1994	Cairo, Egypt	Intellectual Property Rights and Project Evaluation workshop series.
Dr. John Wafula Ms. Stella Muthoni Munyi	July 11-14, 1994	Washington, DC	Intellectual Property Rights.
Mr. Lucas Sese	February 4-17, 1996	Michigan State University Association of University Technology Managers	Intellectual Property Rights and Technology Transfer Internship Program.

Kenya - *Collaboration/Other*			
Dr. Norah Olembo	May 20-25, 1995	San Francisco, California	Present paper on IPR and BIO annual meeting.
Eunice Warimu Jerry Nyagua	April 1993	Stanford University	Participated in Intellectual Property Rights internship.

Indonesia - *Research*			
Dr. Liswidowati	June 24, 1993- June 25, 1995	Dr. Mariam Sticklen Dr. David Douches Michigan State University	Transformation of potatoes with B.t. genes.
Dr. M. Herman Dr. Jeff Lowe	June 1993-June 1995	Dr. Mariam Sticklen Michigan State University	Transformation of potatoes with B.t. genes.
Ms. Amir Bahagiawati	July 3, 1993- June 28, 1995	ICI Seeds, Iowa	Transformation of maize with B.t. genes.
Dr. Anggoro Hadi Permadi	September 12- November 30, 1993	Dr. David Douches Michigan State University	Transformation of potatoes with B.t. genes.
Dr. Anggoro Hadi Permadi	September 22- November 22, 1994	Dr. David Douches Michigan State University	Transformation of potatoes with B.t. genes.
Mr. Saptowo Pardal	September 4, 1994- September 29, 1995	Dr. Jan Tippett ICI Seeds, Iowa	Transformation of maize with B.t. genes.
Dr. M. Herman	November 1995	Dr. David Douches Michigan State University Dr. Maud Hinchee Monsanto	Transformation and regeneration of sweetpotato.

Dr. Anggoro Hadi Permadi	1995	Dr. David Douches Michigan State University	Transformation of potatoes with B.t. genes.
Dr. Minantyorini Abikuano	June-August 1996	Dr. Maud Hinchee	To learn techniques for transgeneration of sweet-potato.
Dr. Asih Karjadi	June-August 1996	Dr. Rebecca Grumet	To learn melon transformation and regeneration, and to perform experiments to develop cucumber transformation and regeneration with American and Indonesian genotypes.
Indonesia - *Biosafety*			
Mr. Adji Atmandjaja Dr. Sutaryo Brotonogoro Dr. Bambang Purwoko	May 10-July 2, 1993	ICI Seeds, Iowa Michigan State University USDA/APHIS, EPA, FDA	Biosafety Internship Program: Guidelines Development
Indonesia - *Intellectual Property Rights*			
Dr. Ati Duriat Mr. Arry Sigit	April 1-28, 1993	Prof. John Barton Stanford Law School	Intellectual Property Rights Internship Program.
Mr. Sisworo Priarso	July 11-14, 1994	Washington, DC	Participated in Intellectual Property Rights Workshop.
Indonesia - *Collaboration/Other*			
Ms. Amir Bahagiawati	June 29-July 2, 1993	Michigan State University	ABSP Technical Advisory Group meeting.
Dr. Sugiono Moeljopawiro Ms. Elda Adiningrat	August 25-26, 1994	Michigan State University	ABSP Technical Advisory Group meeting.
Dr. Firdaus Kasim	October-December 1994	ICI Seeds, Iowa	Attended International Plant Physiology Meeting, and local courses at Iowa State University.
Mr. Edy Listanto	January - September 1995	ICI Seeds, Iowa	Attended International Plant Physiology Meeting, and local courses at Iowa State University.
Dr. Ati Duriat	January 1996	Dr. Rebecca Grumet Michigan State University	To plan collaborative efforts for cucumber transformation.

Publications

Publications of the ABSP Project

BioLink Newsletters

Volume 1.1: Project Overview

Volume 1.2: Focus on Indonesia

Volume 1.3: Focus on Egypt

Volume 1.4: Focus on Capacity Building Activities

Volume 2.1: Focus on Progress by ABSP Private Collaborators, Collateral Research and Policy Work at Other Institutions

Volume 2.2&2.3: Focus on the Changes in the ABSP Management Structure to Facilitate Technology Transfer, and Human Resource Development Activities

Volume 2.4: Focus on Global Workshop and Technology Transfer

Workshop Proceedings

USAID Latin America/Caribbean Region Biosafety Workshop, May 10-13, 1993, Oracabessa, Jamaica

AGERI & ABSP Workshop Series on Biosafety Intellectual Property Rights and Project Evaluation, January 24-31, 1994, Cairo, Egypt

USAID Intellectual Property Rights Workshop, July 11-14, 1994, Washington, DC

Agricultural Biotechnology for Sustainable Productivity Project Mid-Point Report to the Technical Advisory Group, August 1994, East Lansing, MI

Indonesia/ABSP Technology Transfer Workshop, April 18-20, 1996, Cisarua, Indonesia

Articles and Papers

Abdallah, N.A., N.M. Aref, C.M. Fauquet, M.A. Madkour, M.A. and R.N. Beachy. 1993. Nucleotide sequence and genome organization of an infectious DNA clone of tomato yellow leaf curl virus from Egypt. *IXth International Congress of Virology,* Glasgow 8-13 August, P62-13.

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Mariam Sticklen and Heng Zhong. 1994. Method for *In Vitro* Sexual Reproduction of Corn Plants, Patent No. U.S. 5,281,529. January 25, 1994.

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The Scripps Research Institute, ILTAB: A disclosure about the discovery of a possible way to control geminiviruses in general and TYLCV-Eg in particular has been filed, waiting for the transgenic work to be completed. If the transgenic work comfirms the transient sssays a patent would be filed.

Posters

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