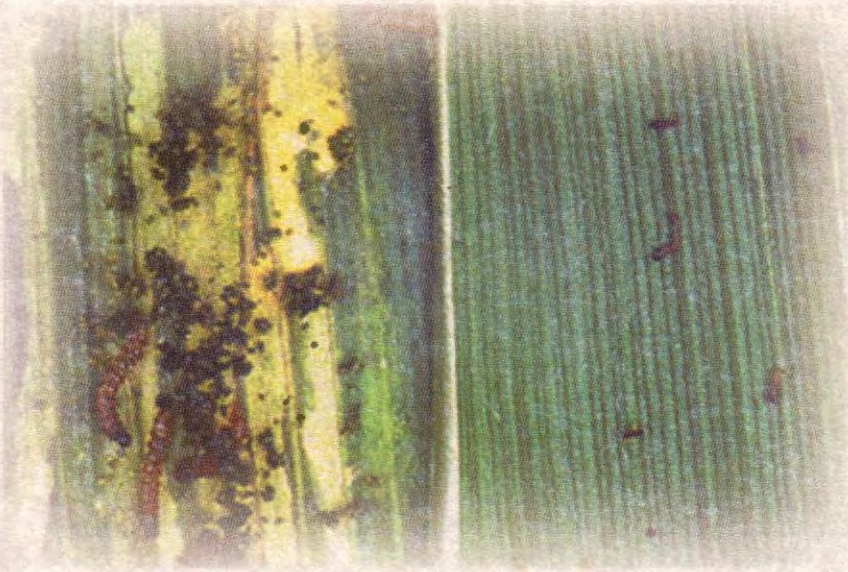
UNDP Global Research and Development Project

GLO/91/014



Reducing Maize Losses to Insect Pests by Enhancing Host Plant Resistance with *Bacillus thuringiensis* Toxin Genes

Final Progress Report

July 1999

CIMMYT

International Maize and Wheat Improvement Center

undp

World Development

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Acknowledgments

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FINAL REPORT ON THE UNDP/CIMMYT PROJECT:

Reducing Maize Losses to Insect Pests by Enhancing Host Plant Resistance with *Bacillus thuringiensis* Toxin Genes

Executive Summary

(Note to readers: Detailed information and/or documentation for the various objectives cited in this report may be found in the respective appendix listed under the heading "Documentation." The appendices may be accessed on the accompanying CD-ROM found on the back panel of this publication.)

Introduction

Maize (*Zea mays* L.) is one of the principal crops in Mexico and other developing countries, where it is used for human and animal nutrition. Insects constitute a major threat to overall production levels of this vital cereal. The importance of insects as a yield-reducing factor increases dramatically when moving from temperate to tropical regions, where much of the developing world's maize is grown. It has been estimated that up to 15% of crops worldwide are lost solely to insect damage; in Mesoamerica, yield losses to insects exceed 30%.

Among the various insect pests, Lepidopteran insects are among the most important in both the industrialized and developing worlds; infestations result in annual losses estimated at over 4 million tons in Brazil and 1 million tons in Mexico. Losses outside of Latin America are also dramatic. Despite the tremendous losses cause by these insects, many developing world farmers do little to control the pests, either because they believe that available measures are inadequate or because they cannot afford chemical insecticides.

Current crop protection methods rely on the use of agrochemicals, which may be both costly for resource-poor farmers to procure and harmful to the environment. The primary bioinsecticide used to control insects is *Bacillus thuringiensis* (*Bt*) var. kurstaki, a bacterium that encodes proteins that are toxic to specific lepidopteran species. *Bacillus thuringiensis* is a species of soil-inhabiting bacteria that is found in most parts of the world. Under certain environmental conditions, the bacterium simultaneously forms a spore and a distinctive crystal, which are the principal sources of the proteins that produce Bt's toxic effects on particular insects. Because a single gene encodes each Bt toxin, it is relatively straightforward to genetically engineer a plant to produce the toxins. The isolation and modification of the gene (termed a "*cry*" gene) that genetically codes for these proteins allows their expression in plants. Among the many advantages to this control method, two were seen by this project's designers as especially important: (1) Bt proteins are highly specific to certain insect species and do not harm non-target insects; and (2) the World Health Organization has determined that these proteins are safe for humans, based on *Bt*'s mode of action.

Following is a synthesis of results from the UNDP-sponsored project "Reducing Maize Losses to Insect Pests by Enhancing Host Plant Resistance with *Bacillus thuringiensis* Toxin Genes" (UNDP Project GLO/91/014). The US$ 5.4 million project, which ran from 1991 to 1998, was based at the International Wheat and Maize Improvement Center (CIMMYT)

located just outside of Mexico City. The overall goals of the project were to use advanced genetic engineering technology from public and private sources to generate tropical maize germplasm that possessed enhanced and durable resistance to major insect pests of the crop (Southwest Corn Borer [SWCB], Sugarcane Borer [SCB], and Fall Armyworm [FAW]) and to provide the improved germplasm to breeders and farmers in developing countries. It was projected that by reducing maize losses to insect pests in tropical agricultural ecosystems, the transformed maize obtained from this project would eventually enhance maize productivity, food security, and reduce the need for pesticides in the developing world. The project also aimed to encourage legal and policy decisions by developing country governments to promote the growth of private sector involvement in agricultural biotechnology. By pursuing an integrated approach that combined applied research, product development, and policy development in the areas of biosafety and IPR, the project sought to assist developing countries with the technology in an environmentally and legally responsible manner.

New technologies will certainly play an important role in enhancing the productivity of the agricultural sector, which will be a fundamental requirement for feeding the world's growing population. To gain access to many of these technologies, countries will need to have appropriate national and institutional policies in place to manage the innovations—this particularly applies to intellectual property rights (IPR). Technology transfer in the agricultural community is changing from informal, free exchange to formal legal agreements. The UNDP/CIMMYT project has taken an integrated approach to the multifaceted problem of technology transfer by combining applied research, product development, and policy development in the areas of biosafety and IPR to assist developing countries in not only accessing and generating technology, but also in using that technology in an environmentally and legally responsible manner.

This project was hailed by external reviewers as one of the most successful biotechnology projects funded by UNDP. Under the auspices of the project, CIMMYT has succeeded in inserting *Bt* (*cry*) genes into tropical maize and in introgressing *Bt* genes provided by the private sector from temperate germplasm into maize cultivars with broad adaptation to the tropics and subtropics. The first wave of CIMMYT transgenic maize has already undergone field testing in Mexico; other materials developed under the project are currently being tested. The project has also facilitated collaboration between CIMMYT and the Government of Mexico in the development of biosafety regulations that govern field testing and the eventual release of transgenic materials.

The achievements cited above confirm that CIMMYT has both the high level technical capability and the strong collaborative relationships with NARSs that are required to successfully execute global maize biotechnology research. The achievements also provide a strong foundation for the second phase of project activity, based on working closely with selected NARSs to move transgenic insect-resistant maize into farmers' fields.

Project Objectives and Functions

The project objectives as stated in the original project document are the following:

1. Identification of at least three *B. thuringiensis* (*Bt*) strains that harbor d-endotoxin genes with effective insecticide action against the major lepidopteran pests of tropical maize.
2. Identification, characterization, and isolation of the genes encoding specific Bt crystal proteins with high levels of toxicity for maize insect pests.
3. Preparation of gene constructs that allow the expression of crystal protein genes in transgenic (maize) plants.
4. Development of appropriate tissue culture technology for regeneration of tropical maize.
5. Introduction and expression of the *Bt* genes in tropical maize plants.

Following the initial meeting of the project's External Advisory Committee (EAC) in 1994, the following objectives were added:

1. Acquire CryIAb transgenic maize from the private sector; evaluate and backcross it into CIMMYT maize lines.
2. Develop deployment strategies for the utilization of transgenic maize.

It was expected that by the end of the project, CIMMYT would obtain fertile maize plants that contained genes encoding the d-endotoxin of *Bacillus thuringiensis* and that expressed sufficient levels of this protein to confer significant resistance to the major lepidopteran insect pests of non-temperate maize genotypes from the CIMMYT maize breeding program. The project team determined that *Bt*-based resistance should be transferred to more resistant materials, which could be used by breeding programs in developing countries and by private companies to generate maize varieties and hybrids of interest to farmers. Collaborations between the public and private sectors could well provide Bt maize for developing country farmers in a more timely manner and at a more reasonable cost than either sector acting alone. Several unanticipated constraints arose during the course of the project, including a legal decision that established proprietary rights over *Bt* genes; the absence of the necessary tissue culture and gene transfer protocols for tropical maize; an inability on the part of developing country breeding programs to utilize the source materials resulting from the project; public and scientific concerns about possible gene flow from transgenic maize to wild relatives of maize.

Documentation

Appendix 2. Project Proposal; Appendix 3. External Advisory Committee Meetings; Appendix 4. Budget.

Project Performance

OBJECTIVE 1.

Identify at least three *B. thuringiensis* strains that harbor d-endotoxin genes with effective insecticide action against the major lepidopteran pests of maize.

Outputs

1.1. The specificity and toxicity of *B. thuringiensis* strains were defined.

Activities

1.1.1. Acquired different strains of *B. thuringiensis.*

1.1.2. Screened and defined the specificity and toxicity of the strains against target pests.

Achievements

The first year of the project was dedicated to identifying the toxicity of the natural *B. thuringiensis* Berliner strains or isolates, as well as the purified toxins of the different Cryl proteins. Twenty-five *B. thuringiensis* strains were selected and defined from a total screening of 426 native isolates, based on their toxicity and effectiveness. It was found that a native Bt strain from Brazil consists of insecticidal crystal proteins that belong to *cry*IB, *cry*IE, and *cryI*Ab genes.

Details

Bacillus thuringiensis is a gram-positive bacterium that produces crystallic protein inclusions during sporulation. These inclusions are formed by proteins called d-endotoxins or insecticidal crystal proteins (ICP), and they vary in quantity and type depending on the strain. The variations in these protein inclusions manifest in terms of their toxicity/non-toxicity to target insect larvae and to non-target insects, plants, and vertebrates. The proteins are completely biodegradable, so no residual toxic products accumulate in the environment.

The proteins exert their toxicity by binding to midgut epithelial cells in the insect larvae, ultimately causing osmotic lysis through pore formation in the cell membrane—a highly effective

mode of action. The analysis of toxic activity through bioassay allows the identification of the powerful toxins against a particular insect.

To isolate novel d-endotoxins from *B. thuringiensis* Berliner, a total of 426 native isolates were screened (in collaboration with CINVESTAV) against four major maize pests: corn earworm (CEW), *Heliothis zea;* fall armyworm (FAW), *Spodoptera frugiperda;* southwestern corn borer (SWCB), *Diatraea grandiosella;* and sugarcane borer (SCB), *Diatraea saccharalis.* As noted, 25 isolates were selected on the basis of high larval mortality against at least one insect species. There was no correspondence among the most toxic isolates when tested against the four different insect species. Although none of the 25 selected strains achieved 100% mortality in any of the bioassays, most of them produced higher toxicities against all four pests than the standard strain HD-1. Of the four insect pests, corn earworm demonstrated both the highest mortality level (96%) and broadest susceptibility to the largest number of isolates (98). None of the other insect species were found to be susceptible at levels above 60% mortality. All of the selected active strains were isolated from stored grain dusts and showed the presence of bipiramidal crystals with Cry I-like proteins, which most of them formed in an associated square (cubic) inclusion, with Cry II-like proteins, according to SDS-PAGE analysis of their parasporal bodies. The most active isolates were included in later studies aimed at identifying putative novel genes.

Efforts to establish insect bioassay capabilities at the Brazilian Agriculture and Livestock Enterprise (Spanish acronym EMBRAPA) in Sete Lagoas, Brazil have largely paralleled CIMMYT's own work in this direction. Insect bioassay activities initially focused on bacterial preparations, but later resulted in the identification of Bt strains that demonstrate good potential activity against FAW. Ms. Fernando Valicente of EMBRAPA visited CIMMYT and CINVESTAV in April, 1993 to learn how CIMMYT performs insect bioassays with partially purified toxins and to learn about toxin purification protocols. Efforts were directed toward screening different Brazilian strains of *Bt*, which were isolated from stored grain dusts, and to purify the toxins from these strains. Four isolates were selected on the basis of 80–100% larval mortality against FAW. The strain #TB8 with 100 % mortality was bioassayed again at CIMMYT and characterized in Alessandra Bravo's Lab (UNAM) at Cuernavaca, Mexico. The characterization was accomplished by enzyme-linked immunosorbent assay using a specific monoclonal antibody against *Cry* toxin and polymerase chain reaction (PCR) analysis using specific primers for different *cry* gene. The results showed that strain #TB8 is unique and that it consists of insecticidal crystal proteins that belong to *cry*IB, *cry*IE, and *cryI*Ab genes.

Documentation

Appendix 5. 1993 Report; Appendix 6. 1994 Progress Report; Appendix 7. 1995 Progress Report; Appendix 11. Sub-contract AB; Appendix 12, Sub-contract RF; Appendix 14. Sub-contract JI; Appendix 15. ECOGEN.

OBJECTIVE 2.

Identify, characterize, and isolate the genes encoding specific Bt crystal proteins with high levels of toxicity for maize insect pests.

Outputs

2.1. Cloned *Bt* genes.

2.2. Data on the toxicity of the crystal protein toxins encoded by individual *Bt* genes from strains of interest and by genes already identified in the work of other research groups.

Activities

2.1.1. Obtained cloned *Bt* genes previously isolated by other research groups.

2.1.2. Isolated *Bt* genes from strains identified under 1.1.; cloned them into vectors; inserted them into *E.coli* hosts.

2.2.1. Tested the toxicity of proteins expressed in *E.coli* hosts on individual Lepidoptera species.

Achievements

Four purified proteins (CryIAb, CryIAc, CryIB, CryIE) showed excellent pest control potential for tropical pests and some African maize borers. Synthetic *cry*IB and *cry*IE constructs were produced. Translational fusions, *cry*IB-*cry*IAB, which allow two Bt toxins to be expressed in a single protein, were also cloned.

Details

An agreement between CIMMYT and ECOGEN was presented and discussed at the EAC meeting, and it was decided that the company would provide CIMMYT access to select Bt strains for screening against various insect species. ECOGEN's subsequent entanglement in a legal suit led the company to cancel its agreement with CIMMYT.

Bioassays with neonate larvae from the four targeted lepidopteran species were performed with the Cry I-type proteins produced by *B. thuringiensis*. Results showed that these proteins are highly specific against the selected pests. The bioassay experiments identified four toxins that were subsequently assigned high priority for the transformation: CryIAb, CryIAc CryIB and CryIE . The LC_{50} for CryIB is 5.21ng/mm^{2}and LC_{50} for CryIAb is 5.14 ng/mm^2.

In addition, the Cry I-type proteins were tested against the following African maize borers: *Sesamia calamistis, Heliothis armigera, Eldana saccharina,* and *Chilo partelus*. CryIB proved to be the most active toxin on *H.armigera*, with an LC_{50} of 4.84 ng/mm^2; followed by CryIAb and CryIAc with LC_{50}s of 18.8 and 22.5 ng/mm^2 respectively. CryIB was the most active toxin for *S. calamistis*, with a LC_{50} of 11.7 ng/mm^2.

Table 1. Cry genes determined most effective against targeted insects

cry Gene	Species
cryIA(b)	*H. armigera, H. zea, S. frugiperda*
cryIA(c)	*H. armigera, H. zea, S. calamistis*
cryIB	*D. grandiosella, H. armigera, S. calamistis, S. frugiperda*
cryID	*S. frugiperda*
cryIE	*S. calamistis, S. frugiperda*
cryIF	*S. frugiperda*
cryIH	*D. grandiosella, D. saccharalis*
cryIIA	*H. armigera, S. calamistis*
Species	***cry* Gene**
D. grandiosella	*cryIB, cryIH*
D. saccharalis	*cryIH*
H. armigera	*cryIA(b), cryIA(c), cryIB, cryIIA*
H. zea	*cryIA(b), cryIA(c)*
S. calamistis	*cryIA(c), cryIB, cryIE, cryIIA*
S. frugiperda	*cryIA(b), cryIB, cryID, cryIE, cryIF*

CIMMYT's collaborations with Centro de Investigación y Estudios Avanzados del IPN (CINVESTAV), Centre de Coopération Internationale en Recherche Agronomique pour le Developpement (CIRAD), and Universidad Autónoma de México (UNAM), directed toward the screening of Bt toxins, proved quite productive as genes providing potential resistance to targeted insects were successfully identified. CIMMYT also obtained some interesting results with Bt maize acquired from the CIBA corporation, however, project researchers observed an apparent lack of resistance to FAW. Meanwhile, several of the *cry* genes bioassayed at CIMMYT and CIRAD demonstrated activity against various insects. This confluence of events led to a research sub-contract agreement between CIMMYT and CIRAD, the aim of which was the production of a synthetic *cry*IB gene construct (with appropriate promoters) for use at CIMMYT.

Results of the bioassays with *cry* gene toxins led to the determination that *cry*IB and *cry* IE genes were effective against many of the targeted maize pests. It was thought that these two toxin recognize different receptors in the insect midgut than the other *cryIA* toxins. To pursue this line of research, a subcontract was developed with Dr. Roger Frutos at CIRAD to produce synthetic *cry*IB and *cry*IE gene contructs for use at CIMMYT. Translational fusion *cry*IB-*cry*IAb, which allows the expression of two Bt toxins in a

single fusion protein, also were developed. Expression of the synthetic gene encoding the truncated protein should result in the production of a Bt protein with an amino acid sequence that is identical to the amino acid full-length native Bt protein.

The first part of the work on this objective was devoted to designing the nucleotide sequence of the synthetic *cry*IB gene. Starting from the published sequence of *cry* IB, the first modification was the substitution of *B. thuringiensis*-preferred codons for plant-optimized codons. The amino acid sequence was used as a template from which the DNA sequence was modified by associating each amino acid with the most preferred codon in plant. Most A-T-rich sequences were removed by adapting the codon usage or through the substitution of C or G to A or T, according to the plant codon usage. The creation of the synthetic gene was accomplished by polymerase chain reaction (PCR) using purified oligonucleotides and a recently developed PCR approach referred to as "Dual-Asymmetric PCR" or "Recursive PCR." A synthetic version of the *Bt cry*IB gene, with a G-C content of 58%, has been produced.

CIRAD prepared a synthetic, or optimized, *Bt cry*IE gene to produce better expression in plants without modifying the aminoacid sequence of the protein. Based on earlier work, in June 1997, CIMMYT decided to synthesize the *cry*IE gene; the gene was supplied to CIMMYT at the end of 1998. The optimized sequence was determined according to the codon usage of the plants (monocotyledons and dicotyledons) and in order to eliminate unwanted A-T-rich sequences, thereby keeping them from interfering with transcription in the plants. The sequence of the wild type *cry*IE gene was used as a basis for determining the modified sequence. The modified sequence uses the codons preferred by monocotyledones and is characterized by the elimination of all interfering A-T-rich sequences. The modification was from nucleotide 1 to 1,840 (amino acid 1 to 612), which corresponds to the sequence coding for the active toxin plus 29 amino acids on the N-terminal end. The full length synthetic *cry*IE gene has been sequenced on both stands by gene walking.

To achieve proper expression, the synthetic genes were inserted into a suitable direct vector in the correct molecular environment. Prior to sequence editing and gene synthesis, it was crucial to identify the promoters and terminators to be used, as well as the selection genes that would be present in the vector. This information allowed the selection of restriction sites, not found in either the regulatory sequences or the selection genes, for cloning the synthetic genes. CIMMYT identified the CaMV 35S promoter with double enhancer and the maize ubiquitin promoter as the most efficient promoters available at that time. The terminator used in the construct was a "classic" terminator, the nopaline synthase terminator.

The project acquired modified *cry*IAb and *cry* IAc genes for its first transformation experiments, as well as information about the relevant vectors that are useful for maize transformation, from the University of Ottawa, Canada. The development of these genes was funded by the Rockefeller Foundation.

Documentation
Appendix 8. 1996 Progress Report; Appendix 12. Sub-contract RF

OBJECTIVE 3.
Develop gene constructs for the expression of crystal protein genes in transgenic maize plants.

Outputs
3.1. Optimal gene constructs for the expression of protein genes in desired plant tissue.

Activities
3.1.1. Evaluated the level of expression of reporter genes under the control of transcriptional regulatory sequences from a variety of different genes.

3.1.2. Developed constructs that contain optimal regulatory sequences with protein genes.

Achievements

More than 37 constructs for both biolistic and *Agrobacterium*-mediated transformation systems were produced, which contained various synthetic *Bt* genes (*cry*IB, *cry*IE, *cry*IAb, *cry*IAc, and a *cry*IAb-*cry*IB fusion) along with suitable markers genes (e.g., *bar*). The *Bt* genes were under the control of either the maize ubiquitin or rice actin promoters, thus allowing optimal expression in maize. "Second generation," or improved, constructs were based on a cassette system allowing simple molecular manipulations for replacement of promoters, genes, etc., as required. A further improvement to the constructs resulted in the use of the bi-directional Maize Gemini Virus (MSV) promoter, which allows expression of two genes at the same time.

Details

The [iiorities in this area of the project's work were the development of suitable optimized vectors for transfer of synthetic *Bt* toxin genes and selection genes into maize and the creation of synthetic *cry*IB, *cry*IE and fusion genes (*cry*IAb-*cry*IB) to produce better expression in plants. The first generation of vectors contained in a single plasmid both the synthetic *Bt* toxin gene and the selection gene(s), based either on pUC18 (Biolaps New England), pGEM7Z (Promega), or BKS (Stratagene) general plasmids bearing an ampicillin-resistance gene. These constructs carry the synthetic *S. viridochromogenes bar* gene (Hoechst) under the control of the CaMV 35S promoter and terminator, and *Bt* gene(s) cloned under the control of maize Ubiquitin or rice Actin promoter.

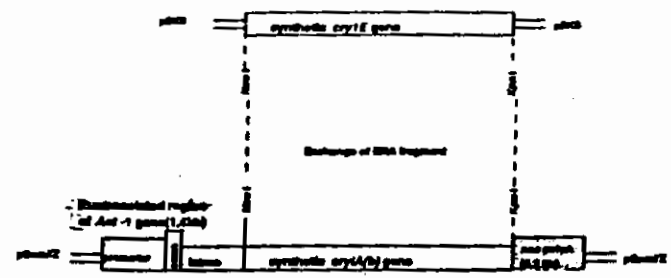
For *Agrobacterium*, "super binary" vectors are based on the use of two separate T-DNAs, the first one containing the antibiotic- or herbicide-resistance gene and the second one containing the trait gene. The first generation of vectors for *Agrobacterium*-mediated maize transformation were based on a binary vector derived from pBIN19, which bears the kanamycin-resistance gene for selection in *E.coli* and *Agrobacterium*. The T-DNA contains the *S. hygroscopicus bar* genes that express under the control of the duplicated CaMV 35S promoter and CaMV 35S terminator and the intron-containing *uid*A gene (gus-intron) under the control of the CAMV 35S terminator and *Bt* gene(s). The first generation of vectors were not flexible and did not allow easy replacement of promoters and genes.

The second generation of vectors for maize transformation, via biolistic or *Agrobacterim* transformation, was based on a more advanced cassette system, which allowed easy and quick replacement of a key element by digestion with a single restriction enzyme that does not recognize cleavage sites elsewhere in the vector (cassette 1—selective gene; cassette 2—sequence controlling toxin gene expression, *Ubi*-1, *Act*-1, or wound inducible promoters; cassette 3—*Bt* gene[s]).

To enlarge the insecticidal spectrum of transgenic maize, a dual strategy was developed: achieve the expression of two different Bt toxin genes in maize and use the bi-directional promoter of the MSV available from CIRAD. Members of the MSV group are single-stranded DNA viruses; mono or bipartite particles. The first strategy employed *cry* gene translational fusion that allow the expression of two Bt toxins through a single fusion protein (CryIB-CryIAb). A bi-directional viral promoter, MSV, which is based on the use of the promoter region of the virus with bi-directional transcription, allowed the simultaneous expression of two trait genes—either two *Bt* toxin genes or a *Bt* gene and a marker gene—within a single cassette, without having to resort to large, inefficient plasmids. These types of vectors met the requirements for both good transformation yield (plasmid size) and enhanced management strategies by expressing two different genes of agronomic interest. (See list of constructs on following page)

Documentation

Appendix 12. Sub-contract RF



1 pGem7Z ACT *cryIA(b) nos ter*

FIGURE _ : Creation of vector containing synthetic *cry1E* gene under the control of the entire 5' unstranslated region (promoter, exon-1, intron-1 and the beginning of exon-2) of rice actin gene Act-1 and the nos terminator.
pBKS *cry1E* was digested by *Nco* I and *Kpn* I and the fragment containing the synthetic cry1E gene was cloned into *Nco*I and *Kpn* I sites of pGem7Z ACT cryIA(b) nos ter plasmid.

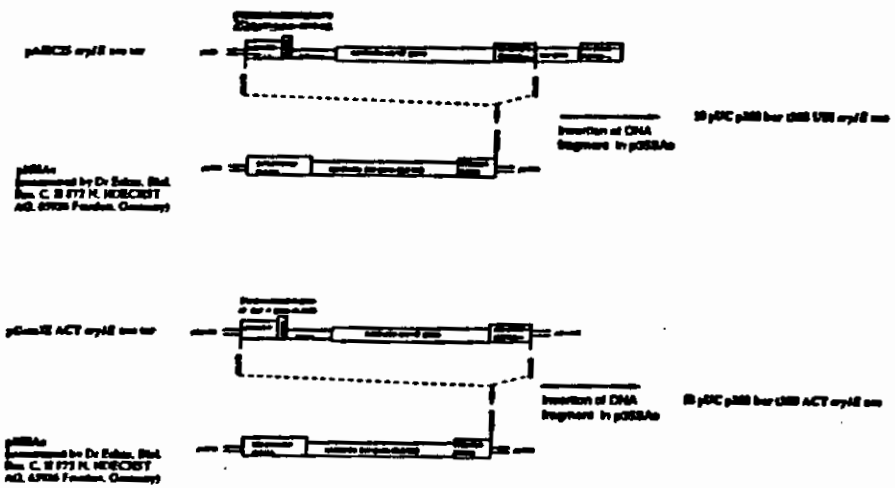
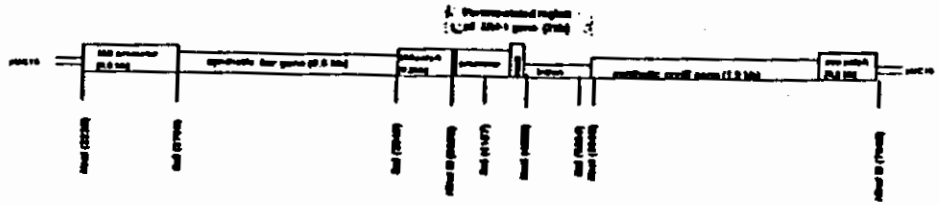
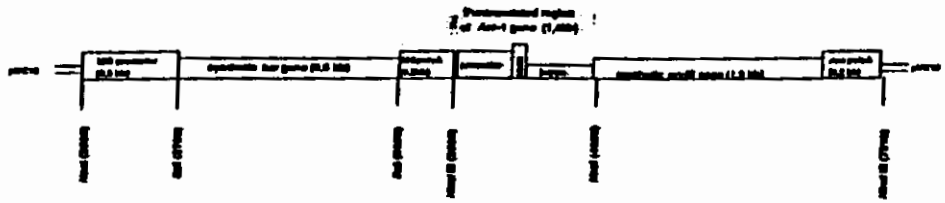


FIGURE _ : Cloning of synthetic *cry1E* gene under the control of different regulatory sequences in tandem with a cassette containing the synthetic *S. viridochromogenes bar* gene under the control of CaMV 35S promoter and terminator.
Each plasmid (pAHC25 *cry1E nos* ter and pGem7Z ACT *cry1E nos* ter) was digested by *Hind*III and each fragment containing both *cry1E* gene and the regulatory sequences was cloned into *Hind*III site of pAc35S.



CONSTRUCT pUC p35S bar t35S UBI cryIB nos



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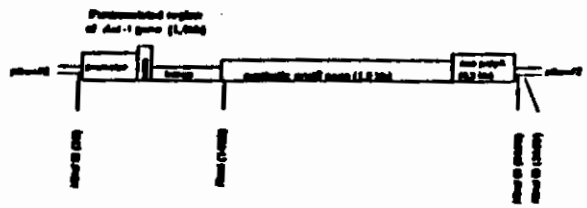

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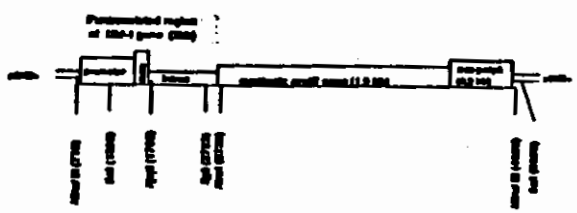

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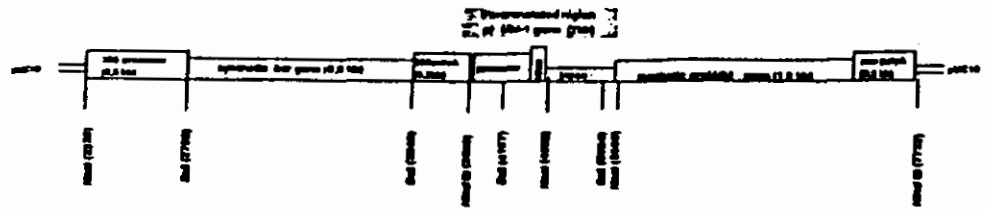

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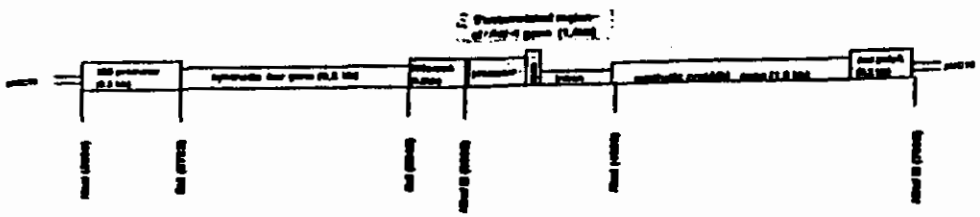

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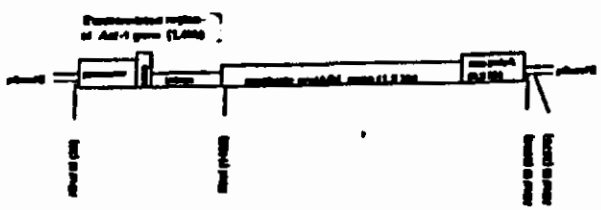

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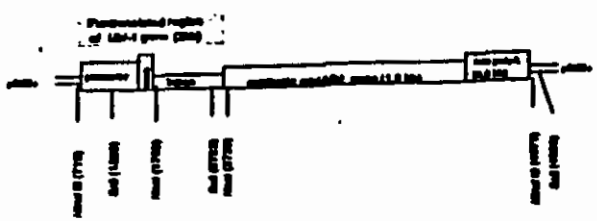

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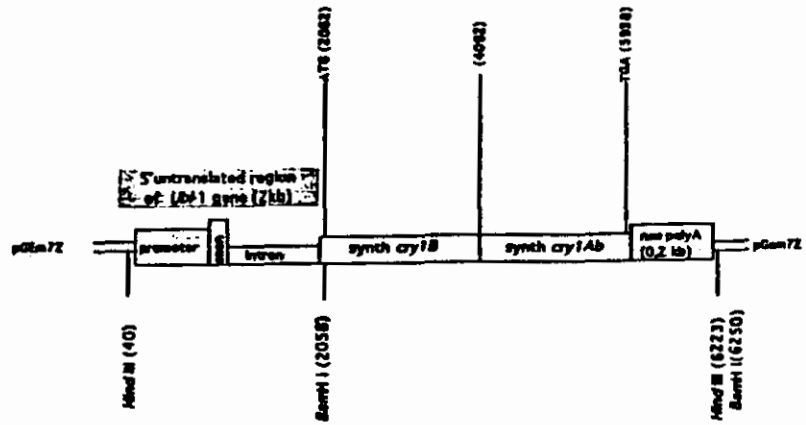
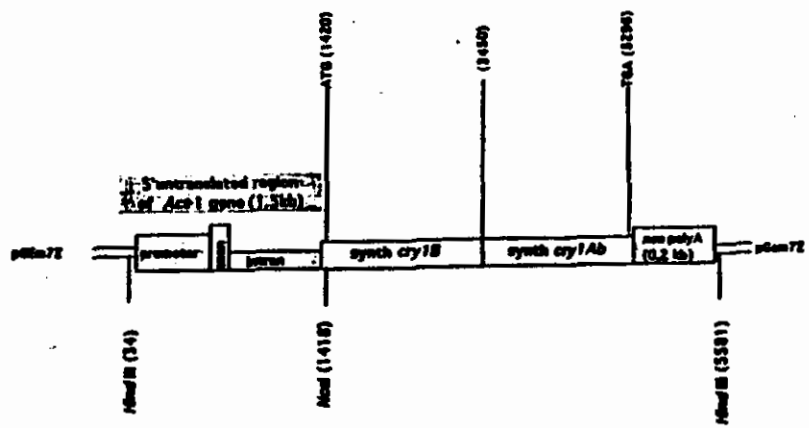


8 pGem7Z UBI cry1B-1Ab nos



2 pGem7Z ACT cry1B-1Ab nos

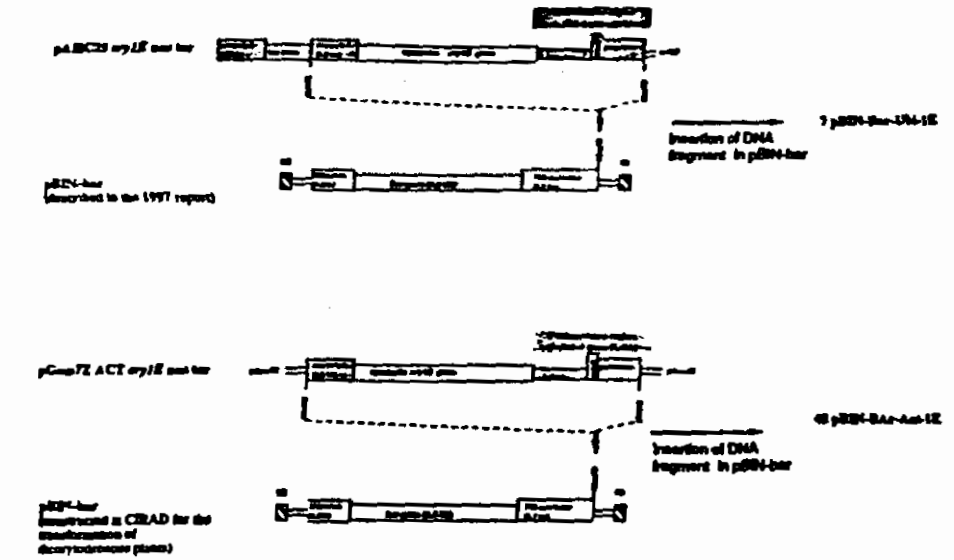
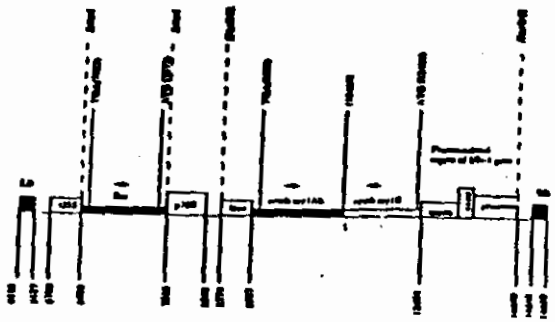
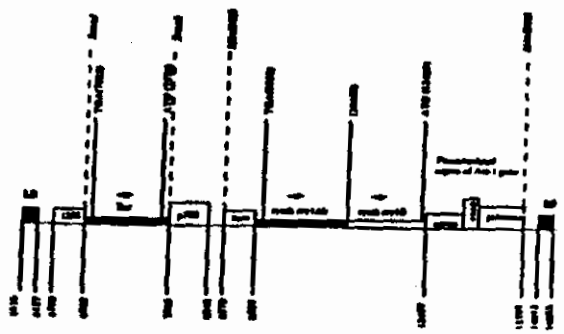


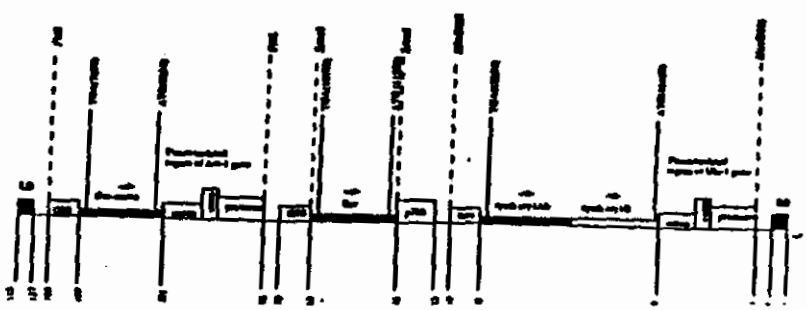
FIGURE ___: Cloning of the synthetic *cry1E* gene under the control of different regulatory sequences in pBIN-bar.
Each plasmid (pAHC25 *cry1E nos ter* and pGem7Z ACT *cry1E nos ter*) was digested by *HindIII* and each fragment containing both *cry1E* gene and the regulatory sequences was cloned into the *HindIII* site of pBIN-bar.



115 pBIN-Bar-Ubi 1E-Ah



27 pBIN-Bar-Act 1E-Ah



133 pBIN-Act GI-Bar-Ubi 1E-1Ah

OBJECTIVE 4.
Develop adequate tissue culture technology for maize.

Outputs

4.1. Highly regenerable maize genotypes for developing country environments.
4.2. Reproducible tissue culture techniques for regenerating desired maize genotypes.

Activities

4.1.1. Selected germplasm representative of developing country mega-environments.
4.1.2. Screened selected genotypes for embryogenic callus formation.
4.1.3. Tested the regenerative capacity of genotypes that displayed a high frequency of embryogenic formation.
4.2.1. Established cell suspension cultures derived from embryogenic calli of genotypes with good regenerative capacity.
4.2.2. Evaluated the somatic embryogenesis and regeneration capacity of the cell suspension cultures.
4.4.3. Optimized culture methods to develop a reproducible protocol for regenerating fertile plants.

Achievements

The plant regeneration potential of 44 tropical and subtropical, 23 midaltitude, and eight highland maize inbred lines was evaluated during the first phase of the project. Embryogenic calli showed a remarkable capacity to regenerate plantlets from 50% of the tropical and subtropical lines, 87% of the midaltitude lines, and 75% of the highland lines tested. Type II callus formation with high potential for plant regeneration from tropical maize genotypes was also reported.

Details

Production of genetically transformed maize depends both on the ability to integrate foreign genes into target cells and the efficiency with which plants are regenerated from genetically transformed cells. Most studies on maize regeneration have utilized genotypes adapted to temperate zones while little attention has focused on the regeneration potential of maize germplasm adapted to tropical and subtropical regions. Immature embryos from elite CIMMYT lines formed embryogenic calli, and plantlets regenerated with high efficiency. CIMMYT inbreds with Antigua origin were among the tropical lines that showed the highest regeneration potential and long-term maintenance of regeneration. Efforts at EMBRAPA in Sete LagoasBrazil have paralleled CIMMYT's own efforts to establish tissue culture technology. Excellent progress has been made at both centers. The screening of Brazilian maize germplasm resulted in several lines being identified for future transformation work. Scientist Carlos Enrique of EMBRABA visited CIMMYT's tissue culture laboratory at during April, 1993 to discuss his efforts at EMBRAPA and to learn the tissue culture techniques being used at CIMMYT. The results from this investigation have been published (Carvalho et al. 1997)

These experiments served as the basis for developing transgenic technology for maize inbreds adapted to tropical conditions.

The transfer of genes for improved trails to "transformable" maize lines requires the subsequent transfer of the inserted transgenes into agronomically acceptable germplasm through conventional crossing . It is often difficult, however, to separate transgenes from linkages to undesirable traits. Thus, it is preferable to apply transformation technology directly to elite breeding lines. Twenty recently-produced elite CIMMYT inbred lines from the center's Maize Program were tested and included the high regenerated lines for transformation experiments.

Although it was proposed that highly regenerable tropical and subtropical inbreds be evaluated in suspension cultures, it was determined that successful transformation of maize was best achieved with immature embryos. Thus it was decided to terminate all efforts with suspension and protoplast cultures and to concentrate on developing efficient methods for the transformation of immature embryos.

Documentation
Appendix 5. 1993 Report; Appendix 6. 1994 Progress Report

OBJECTIVE 5.
Introduce and express the *Bt* genes in maize plants that can be used to transfer the insect resistance to maize lines.

Outputs
5.1. Fertile maize plants with host plant resistance to lepidopteran pests. An effective protocol(s) for transferring and obtaining the expression of alien genes in maize.

Activities
5.1.1. Identified effective transfer protocol(s).
5.1.2. Transformed selected genotypes with the desired *Bt* gene constructs.
5.1.3. Selected and regenerated the transformed cultures into fertile plants.
5.1.4. Tested the transformed plants for the presence and expression of the *Bt* genes.
5.1.5. Evaluated the toxicity of the transformed plants to insects.

Achievements
A biolistic method was successfully used to transform elite tropical maize inbreds, based on the selection and regeneration of immature embryos and calli, from the elite tropical lines CML72, CML216, CML323, CML327, and the hybrids made using those lines. More than 260 events, covering the *cry*IAc, *cry*IB, and *cry*IE genes, were produced and then evaluated. Most of the transgenic plants resistant to the herbicide Basta™ and containing the above cited genes as well as *bar* (*gus*) genes were confirmed by Southern blot analysis. In T3/4, the progenies' transgenic lines demonstrated sexual transmission, Mendelian inheritance, and expression of the introduced genes. In addition, the project scientists also identified factors influencing the effective infection of *Agrobacterium* in tropical maize transformation, and they wrote and published protocol manuals for tissue culture of maize, transformation techniques for tropical maize, and screening of transgenic materials.

Details
The successful production of transgenic plants relies on several essential components: use of regenerable and transformable genotypes; suitable constructs with the most efficient available promoters; easily selected markers; and efficient techniques for transformation, selection, and regeneration. To generate transgenic plants, a biolistic method was established (through a collaboration with Pioneer Hi-Bred) and successfully employed to transform elite tropical maize inbreds, based on the selection and regeneration of immature embryos and calli from elite CIMMYT tropical lines. The project team established a plant genetic transformation system that employed biolistics, which uses high-velocity particles to penetrate cell walls, thereby introducing DNA into intact cells. Each parameter of the system was the subject of individual optimization experiments to improve the conditions for particle bombardment. Optimization was achieved by minimizing tissue damage, leading to higher levels of enhanced transient expression and comparatively stable transformation frequencies. The primary requirement for an optimal target is that the tissue or cells receiving exogenous DNA are culturable *in vitro*, actively dividing, and capable of producing fertile plants. Efficient *in vitro* tissue culture and regeneration systems have formed the basis of transformation studies and reliable protocols have now been developed for tropical maize. Pre-cultured immature embryos or isolated scutella with competent cells for somatic embryogenesis have proven to be excellent targets for microprojectile bombardment and for subsequent rapid recovery of transgenic plants. Compact Type I callus, which can be obtained from the scutellum of immature embryos, is a very good bombardment target for generating transgenic maize in contrast to Type II callus, which was highly recommended at the time of the first maize transformation experiments. Phenotypic, molecular, and biochemical approaches to determine and characterize transgenics and to evaluate gene expression in transgenic plants have been established. To screen a large number of

putative transgenic plants using PCR, the primers for *Bt* and *bar* were designed and synthesized. A reproducible Southern blot analysis was established, using the protocols practiced in the CIMMYT's Applied Molecular Genetics (AMG) laboratory.

To follow up on the promising prospects of specific CryI proteins against four major maize pests, the constructs containing *cry* IAc and *cry* IAb synthetic genes with appropriate promoters and selectable markers were obtained from Dr. Illimar Altosaar of the University of Ottawa, Canada. The transformation experiments continued with constructs carrying *cry*IB and, *cry*IE (provided by our CIRAD collaborators), and recently included the truncated fusion gene *cry*IAb-*cry*IB.

More than 260 events, covering the *cry*IAc, *cry*IB, and *cry*IE genes, were produced and evaluated in CIMMYT's biosafety greenhouses. Most of the transgenic plants resistant to the herbicide Basta™ and containing the *cry*IAc, *cry*IB, *cry*IE, and *bar* (*gus*) genes were confirmed by Southern blot analyses. All T0 plants with *cry*IAc were derived from independent transformation events; the estimated number of copies of intact fragments varied from 1 to 10.

A simple leaf bioassay of To plants, in this instance transgenic tropical maize carrying *cry*IAc and *cry*IB, revealed varying levels of resistance to southwestern corn borer and sugarcane borer. Whole plant assays confirmed the insecticidal activity of these genes, which were expressed in the transgenic progenies. The events demonstrated sexual transmission, Mendelian inheritance, and expression of the introduced *cry*IAc and *cry*IB genes over three to four successive backcross generations. The analysis of transgenics with *cry*IE genes is still in progress.

Pyramiding resistance against a range of insects was obtained by combining insecticidal genes with different modes of action (as part of a project for visiting scientist Willian Blas Cerdan, Universidad Nacional de Trujillo, Peru). Transgenic tropical maize expressing the *cry*IAc and *cry*IB *Bt* insecticidal genes and the heribicide resistant *bar* gene were generated using particle bombardment. Insect feeding bioassays demonstrated the effectiveness of the pyramidal resistance strategy using these insecticidal genes against three critical pests of tropical maize (SWCB, SCB, FAW). Further progeny analyses are in progress.

The key issue to making this vision a reality is transformation efficiency; currently, a large number of transformation events are needed for the selection of a few lines that both carry the selected transgene and display adequate agronomic performance. A major challenge in this area is to increase the efficiency and effectiveness of transformation systems for maize by enhancing and bringing new innovation to current transformation processes. Recently, relatively high-efficiency *Agrobacterium*-mediated transformation of maize was reported with embryo-derived events showing low copy number and simple insertion patterns of maize.

Following up on EAC recommendations, the project team incorporated *Agrobacterium*-mediated transformation into its technological arsenal. During this time, we focused on investigating the factors influencing the efficiency of T-DNA delivery by *Agrobacterium*-mediated transformation. Various factors influence the efficiency of infection with *Agrobacterium*, including different genotypes and explants, preculture medium, *A. tumefaciens* cell density for inoculation, inoculation treatments, inoculation time, co-culture medium, duration and conditions, presence and concentration of acetosyringone at induction, infection and co-cultivation stage, and all of the conditions associated with successful infection.

Two *Agrobacterium* strains—LBA4404 and EHA105—that harbor "super-binary" vectors (pBIN) with two marker gene expression cassettes (*bar*, *gus*) with intron and two *cry* genes (*cry*IAb and *cry*IB) were used for all of the experiments. *Agrobacterium* infection used immature embryos at 3–4 days or 11–14 days precultured on the induction medium and embryogenic callus derived from immature embryos of different tropical lines. *Gus* expression was

detected in most of the tissue after either 2 or 3 days of co-ultivation or after 3–7 days of selection. Highly efficient infection of *Agrobacterium* was observed on both groups of immature embryos 4 days after the initiation medium was used. The defined *gus* spots were present across all of the scutellum surface of freshly isolated immature embryos; most of the *gus* spots were localized on the areas starting to form callus. Our observations showed that higher bacterial density in the suspension and longer inoculation times for the coculture usually yielded more efficient infection and corresponding high *gus* expression on various tissue or cells; however, more cell damage was observed. Transient gene (*gus*) expression was demonstrated within the first two weeks after infection, 4 weeks after selection, and 8 weeks after selection and before regeneration. It was thought that some factor strongly inhibits the regeneration of transgenic cells within the mixture of the tissue, mostly in the stage of regeneration.

Because the technology is at a point where the potential exists to transform virtually any crop-tailored gene, attention is being directed to the establishment of a large-volume of *Bt* transgenics using one of the available techniques. These new transgenic products should provide breeders with another resource for enhancing the genetic composition of their germplasm. However, the actualization of genetically modified plants' potential, particularly tropical maize upon which so many of the world's poor rely, depends on a broad array of non-technical issues, especially, intellectual property rights and related regulatory developments (patent structures and guides for deployment of genetically modified organisms). Release of germplasm containing *cry*IAc genes to CIMMYT clients will require a joint agreement between the University of Ottawa, the Rockfeller Foundation, and CIMMYT. A modified gene for the Bt protein produced by the *cry*IB and *cry*IE genes was synthesized by CIRAD, under contract with CIMMYT; it is owned jointly by CIMMYT and CIRAD.

Documentation
Appendix 6. 1994 Progress Report; Appendix 7. 1995 Progress Report; Appendix 8. 1996 Progress Report; Appendix 9. 1997 Progress Report; Appendix 10. 1998 Progress Report; Appendix 12. Sub-contract RF.

Additional Objectives of the Project

At the time when the project began, many organizations were attempting to produce genetically engineered maize with insect resistance, as well as other traits. However, it soon became evident that only a few companies were capable of actual production. Based on its global mandate for the project, CIMMYT decided to obtain Bt maize lines (which exhibited an effective level of expression) from the private sector. This approach was strongly supported by the EAC and led to the formulation of additional objectives aimed at the development of deployment strategies. The following "additional objectives" and sub-objectives are enumerated separately for ease of explanation. In actual fact, many of the activities listed under an additional objective were conducted either simultaneously or in step-wise fashion with activities cited under other objectives.

ADDITIONAL OBJECTIVE 1.
Acquire CryIAb transgenic maize; evaluate and backcross it into CIMMYT maize lines.
Maize with *cry* IAb genes was obtained by acquiring Bt maize germplasm from the private sector. CIMMYT was given the *cry*IAb gene in homozygous form in an inbred line and in heterozygous form in a hybrid to (1) evaluate the efficacy of plants producing Bt proteins in controlling tropical insect pasts species; (2) gain experience with the regulatory procedures for field testing transgenic maize in Mexico or in other target countries; and (3) establish protocols for moving *Bt* genes rapidly into elite CIMMYT lines. The research agreement stated that the company's *Bt* gene could be transferred into CIMMYT lines by backcrossing them into CIMMYT tropical lines. However, no license was granted to CIMMYT to release the germplasm containing the gene; both parties agreed to negotiate such rights in the future.

We received acquired transgenic maize from a private company in mid-February 1995; the materials were a stably transformed inbred with the *cry*IAb gene, the same inbred untransformed, and a hybrid with the transformed inbred as one of its parents. In the greenhouse, the provided a good level of resistance to leaf feeding by SCB and SWCB compared to the susceptible checks, but no resistance to FAW in the same comparisons.

The acquired transgenic maize was evaluated under artificial infestation for control of SWCB, SCB, and FAW. Eleven tropical inbred lines from CIMMYT's maize breeding program (CML72, CML78, CML216, CML247,CML254,CML270,CML311, CML320, CML321, CML322, and K64R) were backcrossed to introgress the *cry*IAb gene. Both CML123 (white) and CML139 (yellow) lines show some host plant resistance to the African target insects. All BC3F1 seed for the other lines were planted in Tlaltizapan in order to obtain the final backcross; two cycles of selfing in the biosafety greenhouses are now underway. To rejuvenate the seed stocks for use in designated greenhouse and field trials, F1 formations of these CML lines with the line containing the *cry*IAb gene are being undertaken in the biosafety greenhouse.

CML216 has been through four backcrosses with the *cry*IAb gene and is currently in the biosafety greenhouses for selfing in order to fix the gene in question. Two cycles of selfing were needed in order to verify the fixation of the gene and improve the agronomic characteristics. Currently, the CML lines in which the *cry*IAb gene backcrossing has been accomplished are lines that are targeted toward Africa. There is a need for additional lines with the *cry*IAb introgressed for deployment in Asia. Asian CIMMYT outreach has been contacted in order to request potential downy mildew resistant lines with good combining ability to introgress the *cry*IAb gene. Embryo rescue would be used in certain cases to reduce the turn around time on these Asian accessions.

Greenhouse and field experiments confirmed the maize's high level of resistance to the borers (SWCB) and its virtual lack of resistance to FAW. The *cry*IAb gene had a good level of expression in all transgenic backcrosses. It is important to note that leaf damage values corresponded to the average "per entry" of the plants resistant to the herbicide Basta™; plants that were expected to carry the insect resistance gene. The segregation for resistance/susceptibility to Basta™ (and thus to *cry*IAb) was 1:1, as expected. It should be added that both genes co-segregated in all of the plants, i.e., plants resistant to southwestern corn borer were also resistant to Basta™, and viceversa.

Two transgenic hybrids and their isogenic counterparts were evaluated along with six checks under both normal irrigation and drought. On average, there were no significant differences between normal and drought treatments for the first rating of leaf damage taken 14 days after artificial infestation. Under both water regimes, transgenic hybrids registered lower values than non-transgenic hybrids. Protein samples were isolated from leaf tissue (both fresh and dry)of the transgenic entries in the irrigated and drought trails. The two hybrids containing the *cry*IAb transgene presented strong signals of the expected molecular weight of 68 KD and contained approximately 8 ng of CryIAb, which is equivalent to 0.02% of the total soluble protein. In general, Western blot results indicated that (1) either fresh or lyophilized tissues gave similar results; and (2) there is no detectable difference in CryIAb protein concentrations between irrigated and drought trials.

Trials for evaluation of acquired Bt maize with the *cry*IAb gene were also conducted at the International Rice Research Institute's (IRRI) biosafety greenhouses, in collaboration with the Institute of Plant Breeding, University of the Philippines at Los Baños. Whole plant tests of this transgeneic maize demonstrated a high level of resistance against *Ostrinia furnacalis*, the Asiatic corn borer (ACB). Strong interest in Bt maize with the *cry*IAb gene has been shown by the Kenyan Agricultural Research Institute (KARI) and others; possible sites for field trials are Nigeria, Zimbabwe, and South Africa.

Documentation

Appendix 6. 1994 Progress Report; Appendix 7. 1995 Progress Report; Appendix 8. 1996 Progress Report; Appendix 9. 1997 Progress Report; Appendix 13. Sub-contract; Appendix 15. ECOGEN; Appendix 16. Applications; Appendix 17. Field Experiments; Appendix 18. Entomology; Appendix 19. Geneflow workshop; Appendix 20. Geneflow Meeting; Appendix 21. Guidelines.

ADDITIONAL OBJECTIVE 2.

Development of deployment strategies for transgenic maize in developing countries.

The achievements cited earlier in this report strongly support the contention that the CIMMYT has both the technical capacity and the collaborative relationships with national agricultural research systems (NARSs) that are a necessary precondition for deploying transgenic insect-resistant maize in farmers' fields. It follows that the next steps should be to (1) strengthen regulatory frameworks in target developing countries to ensure access to, and the safe deployment of, transgenic material to NARSs and farmers; and (2) foster seed production and technology transfer systems to disseminate transgenic maize varieties to resource-poor farmer. Following the first meeting between EAC and CIMMYT scientists involved in the project, the EAC recommended "an immediate and more proactive role of CIMMYT to interact with appropriate national and international biosafety offices, as well as with the specific communities this technology may impact".

The objectives of a deployment strategy in a developing country should include the following: securing freedom to operate under any dominating patents; acquiring government regulatory approvals for product introduction; economic analysis to determine likely farm-level profitability for different categories of farmers; studies to assess the potential environmental impacts resulting from the deployment of transgenic insect-resistant maize; and monitoring of the transgenics performance in farmers' field.

2a. Explore potential opportunities to promote the development, testing, and utilization of transgenic maize. Investigiate biosafety issues for transgenic maize.

To set the stage for future research on transgenic maize, a CIMMYT Biosafety Committee was established to draft specific guidelines for CIMMYT's research activities and to serve as the regulating body for biotechnology-related activities. The document outlining the biosafety policies of the CIMMYT was developed with the aid of documentation provided by experts in the field and in Mexico's Ministry of Agriculture. The document's policies and procedures will ensure that the products of CIMMYT's research, particularly genetically modified organisms (GMOs), are produced and used safely and that they will not adversely affect agriculture or the environment. The document has three parts. Part One covers the general philosophy and issues of biotechnology and biosafety, and it outlines the responsibilities of the Biosafety Committee and the Biosafety Officer. Part Two provides specific guidelines for those working in the area of biotechnology. Part Three contains a glossary of biotechnology terms, references, and samples of the forms necessary for proper handling of experiments and organisms. The original document was reviewed by the Program Committee of the Board of Trustees of CIMMYT in April, 1994. In addition, application forms for the introduction, use, and release of transgenic materials were produced.

A greenhouse was remodeled and modified to meet biosafety specifications for transgenic work. It was and is accessible only to authorized personnel. Secure cabinets for storage of transgenic maize seed were purchased and placed in CIMMYT's Maize Germplasm Bank, where seed of transgenic material currently not in use is stored under controlled temperature and humidity condition to maintain its viability and vitality.

A small-scale field experiment with Bt maize was conducted at the CIMMYT experiment station in Tlaltizapan, Mexico in 1995. These trials were approved by CIMMYT 's Biosafety Committee, the

National Biosafety Committee of Mexico, and the Mexican Government and were in full compliance with their respective regulatory and biosafety requirements. The second cycle was detasseled and the transgenic plants brought to maturity. This was the first time CIMMYT was permitted to grow transgenic plants to male maturity; the 1999 cycle carried transgenics for self pollination. Field trials were monitored several times by members of the National Biosafety Committee and the Phytosanitary Department (Sanidad Vegetal).

Documentation
Appendix 18. Entomology; Appendix 21. Guidelines.

2b. Explore intellectual property regulatory issues related to the use of transgenic maize.
One major concern of the EAC was the issue of liability in legal disputes over patents and use of transgenic products by investigators in countries that do not have biosafety legislation in place. Another concern was the possible rapid evolution of resistance to Bt proteins due to misuse/ mismanagement of transgenic germplasm. CIMMYT's policy is to not import or test transgenic germplasm in any country without appropriate national biosafety regulations in place.

At the recommendation of the EAC, in 1994, CIMMYT developed a strategy paper regarding the development and deployment of transgenic maize with enhanced insect-resistance based on *Bt* genes. In the paper it was recommended that CIMMYT

1. Ensure that the appropriate patent issues are dealt with to facilitate the free distribution of transgenic maize to cooperators. This includes technology and gene products from both the public and private sectors.
2. Ensure that cooperators are trained and have the facilities to fully comply with their country's biosafety regulations. Provide training for scientists and biosafety officials from those countries that wish to deploy transgenic maize.
3. Investigate with partners how best to manage transgenic maize in tropical ecosystems and develop guidelines for delaying the development of resistant insect populations.

Documentation
Appendix 16. Applications; Appendix 18. Entomology; Appendix 21. Guidelines.

2c. Create management strategies for transgenics to counter the development of resistance to Bt toxins by corn borers.
One major concern regarding the use of insect-resistant transgenic maize is the potential for resistant/tolerant insect biotypes to develop. Resistance to *B. thuringiensis* toxins has been reported in more than 11 species in both field and laboratory studies, demonstrating the need for resistance management strategies to prolong the efficacy of this valuable pest management tool within an integrated control program. Resistance to the *cry*I family of d-endotoxins has already been reported in field and laboratory trials.

Resistance involves reduced binding of toxins to midgut epithelial cells and is generally considered to be a recessive trait. Bt toxins contain two domains: one domain for binding to the midgut and the second domain for insertion into the midgut. Due to the binding specificity of *B. thuringiensis* toxins, each stem borer species of economic importance should be screened against a standard library of *B. thuringiensis* toxins to determine which constructs should be used within a region. Initial screening tests will have to be validated by testing the performance of transgenic lines against various stem borers of economic importance. Studies on the mode of action of *B. thuringiensis* crystal proteins revealed that different crystal protein classes can bind to different receptors present on the membrane of midgut epithelial cells. (Studies are currently in progress.)

In 1994, CIMMYT's maize entomology unit initiated studies to determine the rate at which tropical borers can develop some level of tolerance to Bt toxins. This information will be used in the development of management strategies aimed at prolonging the effectiveness of Bt technology in developing countries. Reaching this objective will require a good

understanding of (1) pest biology, behavior and response to insecticidal proteins; (2) temporal and spatial expression of toxins in transgenic plants; (3) the dynamic of different refugia (areas sown to non-resistant maize where pest population face no selection pressure) strategies in resistance management; (4) the impact of toxin-producing plants on biological controls; and (5) how to deliver the package to resource-poor farmers.

The southwestern corn borer was chosen initially because a healthy colony was available that demonstrated a high degree of susceptibility to CryIAb in comparison to the sugarcane borer or fall armyworm. The methodology used to monitor resistance development differed from previous studies in that transgenic plants (hybrids containing *cry*IAb) were used to apply a 90% selection pressure over each cycle. The rationale for using transgenic plants was twofold: (1) maize plants already have resistance mechanisms in the form of epidermal cell walls, which reduce neonate establishment in mature leaf tissue; and (2) should resistance develop in the field, it will be from selection pressure exerted by transgenic plants. After 14 cycles of selection, the survivorship of the larvae was highly variable, but it did not change significantly.

A small study was conducted to assess the impact of the selection process in developing resistant borer populations. The results showed no larvae surviving after 5 days of feeding on Bt maize. These results are encouraging because under this optimal selection strategy, mild changes did occur in individual vigor during short exposure periods, but the larvae did not complete their development on Bt maize after 10 and 14 cycles of selection for *D. saccharalis* and *D. grandiosella*, respectively. The project identified short duration exposure of neonates to Bt maize (2–48 h) as a technique for detecting early changes in population tolerance to Bt toxins. Increased pupil weight compared to native populations could alert extension and researchers that tolerance to a particular toxin is increasing. This protocol should allow sufficient time to make management changes to reduce selection pressure for a particular toxin in order to prolong the susceptibility of insect pest populations to the suite of available Bt toxins.

To address resistance breakdown, CIMMYT recommends the following:

(1) Verify the effectiveness of *B. thuringiensis* against the targeted pests for a given region. Current thought is that the gene expression should be at a level that produces approximately 20 times the amount of toxin required to kill all susceptible individuals for a given pest species.
(2) Provide a multigenetic barrier to the development of resistance. Develop products containing either two or more *cry* genes with overlapping specificities or a single *cry* gene coupled with host plant resistance developed through conventional breeding.
(3) Develop management strategies that impede resistance development. Strategies will vary among regions, depending on cultivation practices, presence/absence of alternate hosts for insects, germplasm, and existing control strategies for insect pests. Refugia are considered the most important component in a resistance management program.
(4) Provide training for national agricultural systems on the breeding, seed maintenance, and management of transgenic maize.

Documentation
Appendix 7. 1995 Progress Report; Appendix 8. 1996 Progress Report; Appendix 9. 1997 Progress Report; Appendix 18. Entomology; Appendix 21. Guidelines.

2d. Assess the environmental impact of transgenic maize, specifically

— ***Risks associated with distributing Bt maize in developing countries; and***
— ***Maize/teosinte gene flow***

Moving transgenic maize from the lab and greenhouse to the field presents a complicated set of issues that must be resolved. This is particularly true in Mexico, because it is the center of origin of maize and its wild relatives. As it is certain that outcrossing will occur between transgenic maize

and teosinte and maize landraces, two points must be considered and resolved before the field release of transgenic maize in Mexico: (1) the importance that will be assessed to outcrossing of maize transgenics; and (2) the regulations and regulatory framework required to control the release and distribution of transgeneic maize. Similarly, these issues had to be resolved before CIMMYT could field test Bt transgenic maize from the UNDP project.

As an initial response to these issues, CIMMYT facilitated a workshop involving experts in mazie science. CIMMYT was asked to sponsor the workshop by the Mexican National Institute of Forestry, Agriculture and Livestock Research (INIFAP) and the Mexican National Biosafety Committee (CNBA). The workshop, *Maize-Teosinte and Maize-Maize Gene Flow: Implications for Transgenic Maize* was held 25–27 September, 1995.

Briefly, the goals of the workshop were to make concrete recommendations to the Mexican Biosafety Committee on areas of research that needed to be addressed to determine the effects of novel genes introgressing into teosinte and maize landraces and to recommend regulatory guidelines that would allow, at the very least, small-scale field testing of transgenic maize in Mexico by 1996. The two main immediate objectives of this scientific workshop were (1) the establishment of scientific criteria for the sensible regulation of transgenic maize; and (2) the identification of research topics on basic aspects of biosafety and risk analysis regarding the introduction of transgenic maize. Outputs included concrete guidelines for both research involving transgenic maize and risk analysis regarding its deployment. Presentations, conclusions, and recommended courses of action were published in a proceedings document.

This proposed risk analysis was of particular importance in Mexico, because, as noted earlier, it is the center of origin for maize and its wild relative, teosinte. Furthermore, these two species grow in close association with one another in regions of Mexico, which led the directors of the sponsoring institutions to the conclusion that a careful examination of the dynamics of this association is necessary.

Maize/teosinte gene flow

In April 1996, a two-day meeting was held to discuss the possibility of beginning the experiments outlined in the Gene Flow Forum (held in September, 1995). Representatives form CIMMYT, INIFAP, Universidad Autonima del Estado de Morelos, and the Colegio de Postgraduados were involved. The group decided that an experiment to quantify maize to teosinte gene flow should be conducted in an area where teosinte populations have a long history of interaction with maize and where teosinte populations are in no way endangered. It was decided that the experiment would be conducted in Michoacan, near the town of Copandaro, where teosinte is extremely abundant and grows in close association with maize.

The group also decided that isoenzyme work must be conducted in order to identify an allele that is absent in the native teosinte population, but which is fixed in some variety of maize. Eventually, it was decided that single cross hybrids would be the most uniform and vigorous material for the experiment. Subsequently, 46 subtropical and highland hybrids and 17 teosintes were sent to the USDA laboratory at North Carolina State University for isozyme analysis. It was found that the teosinte from Copandaro did not contain isozyme alleles ACP1-3, GOTM-2, PGMA-7, PHI-2, PHI-5. The allele frequency of B-GLU-2 was only 0.8% and for B-GLU, 7.4%. The maize that was sown was a subtropical hybrid from CIMMYT, P43Cameroon-86-1-1-1-1-B-B x CML311. This hybrid has two systems that can distinguish between the maize pollinations of teosinte: GDH R/S, B-GLU 2/7. The Copandaro teosinte had 88% of the regular allele (R) and 12% of the fast allele (F).

Follow up action on the 1996 activities included the planning and implementation of another experiment, near the town of Amecameca in the State of Mexico, to quantify maize to teosinte gene flow. The teosinte growing in the Amecameca area belongs to the Chalco race and was found not to contain isozyme alleles B-GLU-9, EP-Null, MDHA-1

y PGD6B-Null; which are present in the hybrid sown in the experiment (CIMMYT's single cross hybrid CS939113).

Documentation
Appendix 17. Field Experiments; Appendix 18. Entomology; Appendix 19. Geneflow Workshop; Appendix 20. Geneflow Meeting; Appendix 21. Guidelines.

Training for Routine Development of Transgenic Plants

Under the auspices of the project, formal courses were offered to bolster human resource capabilities in transgenic technologies in developing country laboratories and at CIMMYT. Visiting scientists and lab technicians received hands-on training in lab techniques for gene transfer, molecular analysis of transgenics, and relevant fundamentals of the transformation process. The main goal of these trainings was to enable participants to apply tissue culture and transformation technologies for the routine production of transgenic maize. Visiting scientists, who would typically train at CIMMYT for 3–12 months, would return to their home countries and adapt and apply the new technologies and skills they had acquired. CIMMYT scientists would often correspond with these visiting scientists to help backstop and further their efforts.

TECHNICAL STAFF

Biologists from Mexican universities involved in the project were trained in *in vitro* techniques; they also applied the transformation protocols for routine development of transgenic plants.

INTERNATIONAL SCIENTISTS

As part of the project's training component, CIMMYT's Genetic Engineering unit offered a two-week training workshop, "Genetic Engineering of Maize and Wheat," which was sponsored by the UNDP, the Swiss Center for International Agriculture supports (ZIL/SDC), and the Swiss Federal Institute of Technology in Zürich (ETH). The workshop's target group included students and scientists from developing countries who were working in the areas of tissue culture and/or transformation, and who wished to apply transformation technologies to wheat and maize (and other cereal) breeding and research. The workshop was conducted by CIMMYT scientists and some outside experts. The objectives of the workshop were to (1) introduce participants to the fundamentals of tissue culture, relevant to the transformation process in maize and wheat; (2) familiarize participants with all aspects of biolistic-based and agrobacterium-mediated gene transfer systems for maize and wheat; (3) provide theoretical and practical experience in the use of phenotypic biochemical and molecular tools for screening of transgenic plants; (4) discuss issues of transgene stability and expression, biosafety, intellectual property rights, incorporation of transgenics into breeding programs, and ultimate deployment strategies.

Genetic Engineering workshop participants:
Dr. Baldwin C. Chipangura, Zimbabwe; MSc. Edy Listanto, Indonesia; Dr. Eliud Kahiu Ngugi, Kenya; Dr. Guoying Wang, China; MSc. José Ernesto Martínez, Mexico; Ms. Marcia deFátima Schleder, Brazil; MSc. Maria Jose Vasconcelos, Brazil; Dr. Melahat Birsin, Turkey; Dr. Olivia Damasco, Philippines; Ms. Pham Thi Thuy, Vietnam; Dr. Raj Kumar, India; Dr. Rakha Hari Sarker, Bangladesh; Sami Reda Saber Sabry, Egypt; MSc. Shailjia Shrivastava, India; Dr. Shubhada Tamhankar, India; MSc. Wu Ling, China; and Dr. Xu Xiang Yang, China.

TRAINING FOR VISITING SCIENTISTS

Dr. Olivia Damasco—Institute of Plant Breeding; University of The Philippines at LosBaños College of Agriculture, Philippines; three months of training in the transformation of tropical maize.

MSc. María José Vilaça de Vasconcelos—CNPMS/EMBRAPA, Rodavia, Brazil; four months of training in transformation of tropical maize.

Ing. Willian Blas Cerdan—Facultad de Ciencias Biológicas en el Area de Fitogenética Universidad Nacional de Trujillo, Peru; twelve months of training in transformation of tropical maize.

MSc Fernando Valicente—CNPMS/EMBRAPA; Rodavia, Brazil; two months of training in insect bioassay.

MSc Carlos Carvalho; CNPMS/EMBRAPA; Rodavia, Brazil, one month of training in tissue culture of tropical maize.

Documentation

Appendix 22. Genetic Engineering Workshop;
Appendix 23. Genetic Engineering Protocols.

Development of Collaborative Projects with Institutes in Mexico and Other Countries

PROJECT SUBCONTRACTS

A major component of the project was the ability to establish collaborative projects with public and private institutions, as necessary. CIMMYT project team members noted with appreciation that the terms of all of the following collaborations were met and that they proved to be very effective.

GLO/91/014-93b

Production of cloned Cry toxins and bioassays on selected African maize borers

Principle investigator: Dr. Roger Frutos
BIOTROP/IGEPAM/CIRAD
France

GLO/91/014-93b

Production of partially purified toxins from *Bacillus thuringiensis* strains and preliminary analysis of active strains

Principle investigator: Dr. Jorge Ibarra
CINVESTAV
Mexico

GLO/91/014-94a

Production of partially purified and cloned *Bacillus thuringiensis* toxins

Principle investigator: Dr. Alejandra Bravo
Universidad Nacional
Autonoma de Mexico
Mexico

GLO/91/014-94b

Synthesis of the *Bacillus thuringiensis cry*IB toxin gene and development of constructs for transformation of maize

Principle investigator: Dr. Roger Frutos
BIOTROP/IGEPAM/CIRAD
France

GLO/91/014-96a

Development of suitable optimized vectors for transfer of synthetic *Bacillus thuringiensis* toxin genes into maize via particle gun bombardment

Principle investigator: Dr. Roger Frutos
BIOTROP/IGEPAM/CIRAD
France

GLO/91/014-97a

Continuation of GLO/91/014-96a. Development of suitable optimized vectors for transfer of synthetic *Bacillus thuringiensis* toxin genes into maize via particle gun bombardment

Principle investigator: Dr. Roger Frutos
BIOTROP/IGEPAM/CIRAD
France

GLO/91/014-98a

Analysis of binding affinity of *Bacillus thuringiensis* toxins and their potential for receptor competition

Principle investigator: Dr. Roger Frutos
BIOTROP/IGEPAM/ CIRAD
France

COLLABORATIONS

Screen and determine the novel *Bt* genes
Research Agreement between CIMMYT and ECOGEN
USA
(Due to a legal suit, the agreement with ECOGEN was cancelled.)

Development of transformation protocol for tropical maize lines
Pioneer Hi-Bred
USA

Acquired modified *cry*IAb and *cry*IAc genes
Dr. Illimar Altosar
University of Ottawa
Canada

Consultation on the acquisition of materials for use in the project
Dr. Nick Everett
INTERLINK
USA

Provision of seeds of transgenic maize germplasms containing *cry*IAb gene from Ciba Seeds
Research agreement between CIMMYT and Ciba Seeds

Collaboration on tissue culture and insect bioassays.
CNPMS, EMBRAPA
Rodavia, Brazil

Documentation
Appendix 11. Sub-contract AB; Appendix 12. Sub-contract RF; Appendix 13. Sub-contract; Appendix 14. Sub-contract JI; Appendix 15. Sub-contract ECOGEN; Appendix 16. Applications.

PROJECT SUPPORT
The main UNDP contacts with CIMMYT on the project were De. Alva App and Dr. Peter Matlon; the financial representative was Ms. Chinwe Dike.

To provide CIMMYT and UNDP advice and guidance on the project, an External Advisory Committee (EAC) was established in 1994. Committee meetings with UNDP/OPS representatives and project scientists began in the project's second year. Members of the EAC were:

- Dr.Thomas K. Hodges, J.C. Arthur Distinguished, Professor of Plant Physiology, Purdue University, 1155 Lilly Hall of Life Sciences, West Lafayette, USA.
- Dr. Rudolfo Quintero, Head of Bioengineering Department, Universidad Nacional Autonoma de Mexico, Cuernavaca, Morelos, Mexico.
- Dr. Winston Brill, of Winston J. Brill and Associates, 4134 Cherokee Drive, Madison Wisconsin, USA.

Project Staff

Project Director: David Hoisington
Supervisor of Research Operations, Cell Biologist: Natasha Bohorova
Molecular Biologists: Wanggen Zhang, Sarah Fennell
Maize Breeders: Martha Wilcox, Javier Betran, Felix San Vicente, Scott McLean
Maize Entomologist: David Bergvinson
Administrative Assistants: Paul Julstrom, Ogie Bohorov
Laboratory Technician: Bacilisa Luna, Juan-Jose Olivares, Hernandez Rubiceli, Leticia Diaz, Magdalena Salgado, Maria Eugenia Ramos, Mario Pacheco, Pilar Estañol, Rosa Maria Brito
Laboratory Assistant: Alicia Rosaura Almeraya, Hilario Galindo, Maria Asuncion Moreno
Greenhouse Technician: Guillermo Vergara
Greenhouse Assistant: Augustine Aguilar
Secretary: Susana Velazques
Field Assistant: Simon Pastrana

Publications, Presentations, and Other Materials

Journal Publications

Bergvinson, D., and G. Estrada. (1999) Eficacia y Despliegue de Plantas Transgénicas para el Manejo del Barrenador del Tallo del Maíz. *Sintesis De Resultados Experimentales Del Prm 1996-1997* 6: 1-11.

Bergvinson, D., M.Willcox, and D. Hoisington. (1997). Efficacy and deployment of transgenic plants for stemborer management. *Insect Sci. Applic.* 17:1: 157–167.

Blas, W., and N. Bohorova. Co-transformation with different *cry* genes increase plant resistance against tropical pests (in progress)

Bohorova, N.B., B. Luna, R.M. Brito, L.D. Huerta, and D.A. Hoisington. (1995). Regeneration Potential of Tropical, Subtropical, Midaltitude, and Highland Maize inbreds, *Maydica*, 40: 275–281.

Bohorova, N., M. Cabrera, C. Abarca, R. Quintwro, A.M. Maciel, R.M. Brito, D. Hoisington, and A. Bravo. (1997) Susceptibility of Four Tropical Lepidopteran Maize Pest to Bacillus thuringiensis CryI-Type Insecticidal Toxins. *J. Econ. Entomol.* 90 (2): 412–415.

Bohorova, N., W. Zhang, P. Julstrum, S. McLean, B. Luna, RM. Brito, L. Diaz, M.E. Ramos, P. Estanol, M. Pacheco, M. Salgado,.and D. Hoisington. (1998).

Production of transgenic tropical maize with *cryIAb* and *cryIAc* genes via microprojectile bombardment of immature embryos. *Theoretical and Applied Genetics.* (in press).

Bohorova, N., J.E. Ibarra, A.M. Maciel, R.M. Brito, L. Aguilar, and D. Hoisington. (1996) Selection and characterization of Mexican strains of *Bacillus thuringiensis* Berl. active against four major lepidopteran maize pests. *Entomophaga* 41(2): 153–165.

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Carvalho C., N. Bohorova, P. Bordallo, F. Valicente, W. Bressan, E. Paiva. (1997) Type II callus production and regeneration of tropical maize genotypes. *Plant Cell Reports* 17: 73–76.

Hoisington, D.A., and N.E. Bohorova. (1995) Towards the production of transgenic tropical maize germplasm with enhanced insect resistance. In M.Terzi, R. Cella, and A. Falavigna (eds.), *Current Issues in Plant Molecular and Cellular Biology.* Boston, Massachusetts: Kluwer Academic Publishers. 327–331.

Books, Proceedings, and Manuals

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Hoisington D. (1999) Biotechnology for maize and wheat improvement in developing countries: a need, a reality, or a dream? In *Gene and Biotechnology of Food Crops in Developing Countries.* New York: Springer-Verlag Wien. In press.

Wilcox, M. and D. Bergvinson. (1997) Considerations for Bt corn in Mexico. In A. Hruska and M. Lara (eds.) *Transgenic Plants (Bt) in Mesoamerican Agriculture.* Zamorano, Honduras: Zamorano Academic Press. Pp. 98–105.

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Laboratory Protocols

CIMMYT. (1999) CIMMYT Applied Genetic Engineering Laboratory Manual. Mexico, D.F.: CIMMYT.

Presentations

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Bohorova, N., R.D. Hernandes, R.M. Brito, and D.Hoisington. Crecimiento de larvas de tres especies de Lepidopteros en callos de lineas y variedades de maiz suceptibles y resistentes. Presentation at XXX Congreso Nacional de Entomologia, 28–31 Mayo 1995, Universidad Autonoma Chapingo, Chapingo, Estado de Mexico, Mexico.

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Bohorova, N. Plant Transformation Facility, Agronomy Hall, Iowa State University, Ames, Iowa. March, 1996.

Bohorova, N., Lectures: Plant cell and tissue culture; Genes for transformation; Agrobacterium-mediated transformation; and CIMMYT projects in transgenics in the CIMMYT ABC workshop "Genetic Engineering of Maize and Wheat." CIMMYT, el Batan, Mexico. 1998.

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Hoisington D., A.M. Maciel, R.M. Brito, R. Frutos, A. Bravo, J. Ibarra, and N. Bohorova. *Bacillus thuringiensis* genes effective against tropical lepidopteran maize pests. Insect Resistant Maize, 27 Nov.to 3 Dec. 1994, CIMMYT, El Batan, Mexico.

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Documentation

Appendix 24. Manuscripts; Appendix 25. Posters, Presentations

CIMMYT

INTERNATIONAL MAIZE AND
WHEAT IMPROVEMENT CENTER

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CIMMYT home page on www.cimmyt.cgiar.org