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REPORT

(FINAL REPORT)

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Genetic Engineering of Rice for Stem Borer Resistance

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1. EXECUTIVE SUMMARY

At Dhaka University, several Bangladeshi rice genotypes were successfully regenerated from immature embryos. Mr. Sharif Uddin Ahmed, the Bangladeshi project trainee in the Stucklen laboratory, spent three months in Dr. Seraj's laboratory at the University of Dhaka to train a group of young scientists in methods in genetic engineering of rice. A description of project activity at Michigan State University follows.

At MSU the following activities were performed:

- 1) Several plasmids were constructed. These included plasmids containing the synthetic *Bacillus thuringiensis* cryIA(b) driven by different promoters and the potato proteinase inhibitor II gene driven by different promoters.
- 2) Several kill curves were developed to identify the maximum levels of G418 and PPT selectable marker chemicals for rice transformation. As a conclusion, PPT was found most effective for use in rice transformation studies.
- 3) Rice immature embryos were grown in a greenhouse, harvested, and bombarded with plasmids containing the Bt and proteinase inhibitor genes. These embryos were also bombarded with either plasmid containing the bar selectable marker or NPT II genes controlled by 35S promoter in a co-transformation fashion. Callus lines were selected using the optimal levels of G418 or PPT. Over 500 putatively transgenic plants were regenerated.
- 4) Molecular analysis (PCR and Southern blot hybridization) of the putatively transgenic plants have shown that several of these plants are transgenic. Second generation plants were produced in our greenhouses. Southern blot and western blot were conducted to confirm the stability, as well as the level of expression of foreign genes in R₁ rice plants.
- 5) Insect feeding of transgenic plants.

Since it is illegal to import rice major insects to USA, we planned to use insects that are already in USA in our bioassay studies. Bioassays of putatively transgenic plants against *Ostrinia furnacalis*, *Spodoptera frugiperda* and *Nilaparvata lugens* has started. Although some of the R₀ transgenic rice plants showed certain degree of resistance to these insects, no difference was seen statistically between transgenic and the untransformed plants at the laboratory feeding level.

2. SECTION 1

2.1. Research objectives

Our long-term goals have been human resource development (training of Mr Sharif Ahmed, a scientist from Bangladesh, in the techniques of rice transformation) and to evaluate the possibilities of producing stem borer resistant rice plants by genetic engineering To attain these goals, we have directed our efforts toward the following specific objectives

- 1 Obtain a synthetic bt gene from a private sector for transformation of rice
- 2 Construct a series of mini-genes containing this synthetic bt gene controlled by different promoters
- 3 Transform rice cell lines (using an accelerated tungsten bombardment device) with plasmids described in objective 1, and regenerate plants
- 4 Confirm the integration of the genes at the genomic level and the expression of gene at the mRNA and protein level in putatively transformed rice plants

In addition to the objectives initially proposed in our proposal, we have also performed studies on insect feeding on transgenic plants

2.2. Research accomplishments

- 1 System development at the University of Dhaka was continued and seven Bangladeshi rice varieties were regenerated from mature and immature embryos During the summer of 1994, when Mr Ahmed returned to his institution to train his fellow scientists for three months, the most regenerable Bangladeshi rice varieties were used in Dr Seraj's laboratory for transformation studies Since no Biolistic gun is available in that laboratory, Mr Ahmed and Dr Seraj examined possibility of using the silicon carbide method to transform rice with a Gus gene regulated by rice actin promoter Unfortunately, the silicon carbide for transforming rice was found to be very inefficient as compared to the Biolistic method

Bangladeshi rice varieties used in regeneration studies included *Oryza sativa* L (indica) Var, Binnatoa BR 1, BR 3, BR 14, BR 7, BR 21, BR 22, BR 24, and Purbachi (a local variety) These rice varieties are inexpensive and mostly high

yielding Among all three varieties of BA, BR7, and BR12 were most regenerable from both immature and mature embryos

2. Plasmid constructions and plant transformation

Construction of plasmids/mini-genes

Seven plasmids containing the synthetic *Bacillus thuringiensis* cryIA(b) driven by different promoters were constructed from fragments coming from plasmids pCIB4418, pCIB4421, pTW-a and pDM302 The constitutive promoters of cauliflower mosaic virus 35S (35S 5') and rice actin1 (Act1 5'), and the tissue specific promoter of maize phosphoenol pyruvate carboxylase (PEPC 5'), were used to express the synthetic cryIA(b) All the constructs are transcriptional fusions and have the following promoters for synthetic cryIA(b) pRES7193 - 35S 5' (two expression cassettes per plasmid molecule), pRES7293 - tandem Act1 5'-35S 5', pRES7493 - 35S 5', pRES7793 - tandem Act1 5'-PEPC 5', pRES7393 - PEPC 5', pRES107 - tandem Act1 5', and pRES108 - Act1 5' The cryIA(b) expression cassette in all the constructs has PEPC intron #9 and 35S terminator pRES7193, pRES7393, and pRES7493 also contained the potato proteinase inhibitorII (pin2) driven by its wound inducible promoter and terminator The plasmids ranges from 6.23 to 14.63 kb and can be used as closed circular or linear DNA fragments in transformation experiments

Expression cassettes of synthetic cryIA(b) driven by 35S and PEPC promoters were also subcloned to *Agrobacterium* binary vector pBIN19 and designated as pHSE201 and pHSE202, respectively The constructs can be introduced into hypervirulent strains of *A. tumefaciens* that infect monocot

3. Regeneration and transformation of rice

As reported earlier, three rice genotypes (Basmati, IR8, and Fujisaka) were chosen for Dr. Stucklen's laboratory These genotypes were selected so that the Bangladeshi scientist can obtain training on rice regeneration/transformation As reported earlier, during year 1 of the project, Dr. Stucklen's laboratory developed an efficient in vitro regeneration system for transformation of these genotypes Also, kill curves were developed to identify an optimal level of selectable marker (PPT and G418) for each rice variety

Fujisaka 5 callus lines were co-transformed by microprojectile bombardment of embryogenic calli and immature embryos with different plasmid constructs containing the synthetic *B. thuringiensis cryIA(b)* and *pin2*, and plasmids containing the selectable marker *bar* which confers resistance to PPT or glufosinate ammonium. The appropriate concentration of glufosinate ammonium for the selection of transformed calli was 5.0 to 5.5 mg/l. Higher concentrations were found to be more effective in inhibiting the growth of untransformed resistant cell clusters but reduce the regeneration capability of the transformed calli. A total of 316 plants were regenerated from 3400 bombarded calli and immature embryos in 28 independent experiments. Sixty five of these plants were positive for synthetic *cryIA(b)* by PCR, 46 of which showed positive signal in slot blot analysis. Three putatively transgenic plants (Plant 117, 132 and 187) were identified by Southern blot analysis. Second generation plants from these transformants were positive for the presence of synthetic *cryIA(b)* by PCR. Two plants from 187 and one from 117 were positive in western blot analysis. Several other plants were also positive in Southern blot hybridization but smaller fragments were also noted in the undigested DNA, which might indicate the presence of extra-chromosomal DNA.

Several of the plants were positive for the presence of the *bar* coding sequence but no hybridization at high molecular weight undigested DNA was noted. All the plants did not survive when sprayed with 1.0% Ignite (20% glufosinate ammonium) although some survive at 0.7% Ignite.

Molecular analysis to confirm integration and expression of genes in plants

Basmati 370 and Fujisaka 5 transformed using a plasmid containing the *pin2* driven by rice *Act1* promoter. First generation plants were positive in PCR and slot blot analyses. Southern blot analysis also gave positive results but hybridization in undigested genomic DNA was noted as distinct bands at low molecular weight, that might indicate the presence of extrachromosomal DNA. Second generation was developed from transgenic rice plants. Few of the R1 plants also showed low molecular weight bands, indicating the possibility that the gene has been integrated.

Insect bioassay

Since it is illegal to import rice major insects to USA, we planned to use insects that are already in USA in our bioassay studies. Bioassays of putatively transgenic plants against *Ostrinia furnacalis*, *Spodoptera frugiperda* and *Nilaparvata lugens* has started. Although some of the R0 transgenic rice plants showed certain degree of resistance to these insects, no difference was seen statistically between transgenic and the untransformed plants at the laboratory feeding level.

2.3. Scientific impact of collaboration

The impact of the research on human resource development, including training of the Bangladeshi fellow (Mr. Sharif U. Ahmed) in the U.S., and his training of other Bangladeshi scientists in Bangladesh during the summer has been the main focus of this research collaboration. Also, the accomplishment of Dr. Seraj in regenerating Bangladeshi varieties of rice and the U.S. laboratory in transforming rice is very important. We believe that we have transformed R0 Basmati rice plants for the first time. The fact that this rice genotype is susceptible to rice stem borers while being the most aromatic, expensive, and desirable Indica rice at the global level makes our research findings most attractive.

2.4. Description of project impact

Our close interaction including two three-day meetings, transfer of scientific information to the University of Dhaka, has had a definitive impact upon our collaborators' performance in regeneration of rice plants from immature embryos. The Bangladeshi trainee has learned all of the techniques for genetic engineering of rice and has transferred techniques to his institution in Bangladesh. Using a bt gene used for research only.

2.5. Strengthening of developing country institution

A set of supplies were sent and more supplies have been purchased to be shipped to the developing country partner, Dr. Seraj. The exchange of protocols, two personal meetings, and the training of Mr. Ahmed at Michigan State University.

have been among the original objectives of the project

Beyond the scope of original proposal, and with the approval of the USAID officer, the travel of Mr Ahmed and his subsequent training of members of Dr Seraj's research staff at the University of Dhaka has also strengthened the knowledge and expertise of the developing country collaborator

In summation, the main investment of the collaborative effort has been on the training of the Mr Sharif Ahmed, the Bangladeshi scientist, in the Sticklen laboratory and transfer of technology through the sending of protocols by fax and mail to Dr Seraj in Bangladesh

3. SECTION 2

3.1. Managerial issues

Since no management funds were identified in the proposal, the scientific management of the project has been performed by the PI (Dr Mariam Sticklen) At the University of Dhaka, the project has been managed by Dr Seraj The management of the budget of the entire project has been performed by the MSU Office of Contracts and Grants There has been an excellent relationship among all parties

3.2. Special concerns

No concerns The project has progressed with the excellent support and cooperation of the USAID project officer

3.3. Collaboration, travel, training and publications

Collaboration

Under the auspices of the USAID/SCI, MSU has established an exceptional collaborative effort with the University of Dhaka in Bangladesh Based on this collaboration, a Bangladeshi scientist obtained training at MSU Routine scientific communication is taking place between MSU and Dhaka University

Travel

1 Dr Seraj from Bangladesh and Dr Sticklen from the U S traveled to The Rockefeller Foundation Rice Biotechnology conference last spring Since no budget was available for such a trip in the project, travel expenses were mainly paid by the Rockefeller Foundation and partially by Michigan State University

2 With the approval of the USAID project officer, Mr Sharif Ahmed traveled from MSU to Dhaka for three months to train scientists in Dr Seraj's laboratory Expenses and salaries were paid from the Bangladesh portion of the budget He also transferred some chemicals and supplies to Dr Seraj's laboratory

3.4. Request for AID or BOSTID action

We are pleased with the excellent AID/BOSTID support and prompt actions as related to our collaborative work with Bangladesh