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Genetic Transformation of the Cocoa Tree, *Theobroma cacao L.*

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Executive Summary

Cocoa beans (seeds) are a $3 billion cash crop for developing countries in the tropics, second only to coffee. Because of the importance of the cocoa tree, *Theobroma cacao*, research institutions in cocoa-producing countries have been trying to breed for resistance to diseases and insects, which can destroy 10 to 30 percent of the cocoa crop each year (3,6). Cocoa breeding, however, is slow, expensive, and requires much land. Another way disease and insect-resistant plants can be created is by introducing foreign genes conferring resistance to these pests, i.e. by genetic engineering. The first step in this process is to develop an efficient way to introduce foreign DNA into the cocoa tree.

Thus, experiments were initiated to genetically transform the tree. We succeeded in genetically transforming cocoa leaf cells using the soil bacterium *Agrobacterium tumefaciens*, as demonstrated by biochemical and molecular analyses. Because transformation frequencies were low and plants could not be regenerated from transgenic cells, we are now attempting to create transgenic somatic embryos by high velocity microprojectile-mediated gene transfer (biolistics) and to regenerate transgenic plants from these embryos.

In addition to Dr. K. K. Oduro, the co-principal investigator on this project, other scientists and students from developing countries have been involved in this project. Dr. Yaw Adu-Ampomah, a Senior Research Scientist at the Cocoa Research Institute of Ghana worked for six months in the American Cocoa Research Institute (ACRI) Molecular Biology Laboratory at Penn State University to learn plant genetic transformation and molecular biology techniques. He will be performing genetic transformation experiments in Ghana. Mr. Abdoulaye Traore, a graduate student from the Ivory Coast, is working on creating transgenic cocoa calli and trees resistant to mirids, the major insect pest of cocoa in West Africa. The experimental tissues that Mr. Traore creates will be sent to Dr. Coulibaly Nanga, Director of the Coffee and Cocoa Department at IDEFOR in the Ivory Coast, who will test them for their ability to kill mirids. Dr. Asril Darussamin, Director of the Indonesian Biotechnology Research Institute for Estate Crops, will be collaborating with the ACRI Lab on creating transgenic cocoa trees resistant to pod borer, the major insect pest in South-East Asia.

Although funding from the USAID grant has terminated, experiments are still being continued and scientists from developing countries are still being trained. Funding is now being provided by the ACRI Lab and by additional funding from ACRI.

Research Objectives

The purpose of conducting this project is to develop an efficient genetic transformation system for cocoa so that the tree can be engineered for resistance to its major diseases and insect pests. By creating disease and insect-resistant plants, the income of cocoa farmers will increase, less land will be required to produce the same amount of cocoa beans, and pesticide and fungicide use will decrease.

This projects complements the ongoing research of all cocoa researchers that are breeding for resistance to diseases and insects because we will be providing novel genetic material for breeding programs. With regard to genetic engineering of cocoa, we are the most advanced; no other research group in the world has genetically transformed cocoa cells.

In addition to the USAID grant, this project has been supported significantly by several organizations: 1) the American Cocoa Research Institute by providing all of the research facilities through an endowment to Penn State University and by providing funds for a full-time research technician just to work on genetic transformation, 2) the Penn State University Office of International Affairs for providing funds for a Plant Molecular Workshop at the University of Ghana in 1992 and by providing one-month support for Dr. Yaw Adu-Ampomah to work in the ACRI Lab for six months, 3) the International Human Frontiers in Science Program Organization (France) for providing three-months support and travel expenses for Dr. Adu-Ampomah to work in the ACRI Lab., and 4) the Ivory Coast government for providing a scholarship to Mr. Abdoulaye Traore, a graduate student in the ACRI Lab working on this project.

Methods and Results

The introduction of a foreign test gene - one conferring plant resistance to the antibiotic kanamycin - into cocoa leaf cells using *Agrobacterium tumefaciens*-mediated gene transfer and analyses confirming integration of foreign DNA are described in the enclosed publication.

Impact, Relevance and Technology Transfer

The purpose for creating transgenic cocoa trees is to address some of the many problems encountered with the cultivation of cocoa, the second most important cash crop for developing countries. Overall, from 10 to 30 percent of the potential worldwide cocoa crop is lost each year to diseases and insect pests. Therefore, our

long-term objective is to apply advances made in engineering other plants for disease and insect resistance to the cocoa tree. For example, two highly successful projects to genetically engineer plants for resistance to viral diseases and Lepidopteran insects (larvae of moths and butterflies) have been to express viral coat proteins and insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* (*B.t.*) in transgenic plants (reviewed by 7 and 9). By applying this technology to cocoa, we will be able to create trees resistant to swollen shoot virus, the major disease limiting cocoa production in West Africa (esp. Ghana) (6), and resistant to pod borer, the major insect pest of cocoa in South-East Asia (esp. Indonesia and Malaysia) (3). Many other strategies for controlling diseases and insects by genetic engineering are also being investigated (1,2,4,5,8), and once an efficient genetic transformation system is established for cocoa, these advances can be applied to the tree. Ultimately, the result of our research will result in increased cocoa yields and income for farmers, and reduced reliance on fungicides and pesticides.

This project will complement ongoing cocoa research programs throughout the world by providing new genetic resources for breeders. Because of the 3 to 5-year generation time, a complex self-incompatibility system, and the large size of the trees compared to herbaceous plants, cocoa breeding is slow, complicated, and expensive. Thus, every cocoa-growing region in the world could benefit from our work.

Long-term collaborations between the ACRI Molecular Biology Lab and institutions in cocoa-producing countries have been established. We have provided training, *Agrobacterium* strains, and protocols to Dr. Adu-Ampomah of the Cocoa Research Institute of Ghana. We have established a collaboration with Dr. Asril Darussamin, Director of the Indonesian Biotechnology Research Institute for Estate Crops, to create and test transgenic cocoa cells and plants expressing *B.t.* insecticidal crystal proteins for their activity against cocoa pod borer. The Ivory Coast Ministry of Agriculture provided a scholarship for a graduate student, Mr. Abdoulaye Traore, to work in the ACRI Lab. Mr. Traore will be introducing protease inhibitor genes into cocoa for the purpose of engineering the tree for resistance to mirids. His experimental material will be sent to Dr. Coulibaly Nanga in the Ivory Coast for testing. Once we have developed an efficient genetic transformation system for cocoa, other collaborations with researchers in cocoa-producing countries will be established.

The scientific capabilities of collaborating countries have been improved by having scientists from collaborating countries work in the ACRI Lab and by equipment and supplies sent to Dr. Oduro's laboratory at the University of Ghana.

Project Activities/Outputs

Training and Collaboration:

1) Dr. K. K. Oduro worked in the ACRI Lab for six months in 1992 learning cocoa tissue culture and genetic transformation techniques. Equipment and supplies were sent to his laboratory at the University of Ghana.

2) Dr. Yaw Adu-Ampomah, Senior Research Officer and Head of the cocoa tissue culture facility at the Cocoa Research Institute of Ghana, worked in the ACRI Lab for six months in 1993. He will be performing genetic transformation experiments in Ghana using the vectors and techniques developed as part of this grant. Funding for his travel and support were provided by the International Human Frontiers in Science Program Organization, the ACRI Lab, and the Penn State Office of International Affairs.

3) Mr. Abdoulaye Traore, a student from the Ivory Coast, began his Ph.D. studies in the ACRI Lab in January 1994. He is continuing with the genetic transformation studies and will be creating transgenic calli and plants resistant to mirids. He is supported by the Ivory Coast Ministry of Agriculture.

4) Dr. Asril Darussamin, Director of the Indonesian Biotechnology Research Institute for Estate Crops, visited the ACRI Lab for one week in December 1994 to become familiar with the projects and capabilities of the ACRI Lab. Furtek and Dr. Asril have agreed to collaborate on a project to engineer the cocoa tree for resistance to pod borer.

5) Dr. Coulibaly Nanga, Director of the Coffee and Cocoa Department at IDEFOR in the Ivory Coast, will be collaborating with us on a project to engineer the cocoa tree for resistance to mirids.

6) Dr. Steven Sain, the postdoctoral scientist working on this project in the ACRI Lab, left in December 1993 for a permanent position. Half of Sain's salary was provided by the USAID grant and the other half was provided by supplementary funds from the American Cocoa Research Institute.

Workshop:

In November 1992, the University of Ghana and Penn State University sponsored a Plant Molecular Biology Workshop at the University of Ghana. Funding ($10,000)

was provided by the Penn State Office of International Affairs. Furtek lectured on genetic transformation of plants and taught several laboratories.

Publication: A manuscript entitled "Genetic transformation of cocoa (*Theobroma cacao*) leaf cells using *Agrobacterium tumefaciens*" by S. Sain, K.K. Oduro, and D.B. Furtek has been published in Plant Cell, Tissue and Organ Culture (1994, Vol 37, pp. 243-251) (enclosed).

Project Productivity

Although we were able to introduce foreign genes into cocoa cells using *A. tumefaciens*, transformation frequencies were low and the cells that were transformed were from leaves and not embryogenic. After these experiments were concluded, we obtained two cocoa plant regeneration procedures that are now being adapted for our transformation studies. During various stages in plant regeneration, cells will be transformed by high velocity microprojectile bombardment using a biolistic system purchased from Biorad, Inc. in April 1994. The regeneration process will be continued normally, except that media will contain an antibiotic to select for transgenic cells.

Future Work

Although the USAID grant has terminated, developing a genetic transformation system for the cocoa tree is still the highest priority in the ACRI Lab. Because transgenic cocoa calli can be made using *A. tumefaciens*, we will soon be generating cocoa calli expressing *B.t.* insecticidal crystal proteins and protease inhibitor genes. Calli expressing *B.t.* insecticidal crystal proteins genes will be sent to collaborators in Indonesia (Dr. Asril) and Malaysia (collaborator not yet determined), who will then test them against cocoa pod borer. Calli expressing protease inhibitor genes will be sent to the Ivory Coast (Dr. Nanga), where they will be tested against mirids. Concurrently with these experiments, we will be developing a method to regenerate plants from transgenic cells. Once transgenic cocoa plants can be made reliably, we will create transgenic plants expressing *B.t.* insecticidal crystal proteins and protease inhibitor genes, as well as genes conferring resistance to fungal pathogens.

Literature Cited

1. Boulter, D., Gatehouse, A.M.R. and Hilder, V. 1989. Use of cowpea trypsin inhibitor (CpTI) to protect plants against insect predation. Biotech. Adv. 7: 489-497.

2. Cornelissen, B.J.C. and Melchers, L.S. 1993. Strategies for control of fungal diseases with transgenic plants. Plant Physiol. 101: 709-712.

3. Entwistle, P.F. 1987. Insects and cocoa. In: Cocoa. 4th ed. Wood, G.A.R. and Lass, R.A. pp. 366-443.

4. Gatehouse, A.M.R., Shi, Y., Powell, K.S., Brough, C., Hilder, V.A., Hamilton, W.D.O., Newell, C.A., Merryweather, A., Boulter, D. and Gatehouse, J.A. 1993. Approaches to insect resistance using transgenic plants. Phil. Trans. Royal Soc. London: Ser. B-Biol. Sci. 342: 279-286.

5. Hain, R., Reif, H.-J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P.H., Stocker, R.H. and Stenzel, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature. (Lond). 361: 153-156.

6. Lass, R. A. 1987. Diseases. In: Cocoa. 4th ed. Wood, G.A.R. and Lass, R.A. pp. 265-365.

7. Llewellyn, D., Cousins, Y., Mathews, A., Hartweck, L. and Lyon, B. 1994. Expression of *Bacillus thuringiensis* insecticidal protein genes in transgenic crop plants. Agric. Ecosys. Environ. 49: 85-93.

8. Melchers, L.S., Sela-Buurlage, M.B., Vloemans, S.A., Woloshuk, C.P., Van Roekel, J.S.C., Pen, J., van den Elzen, P.J.M. and Cornelissen, B.J.C. 1993. Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. Plant Mol. Biol. 21: 583-593.

9. Strittmatter, G. and Wegener, D. 1993. Genetic engineering of disease and pest resistance in plants: present state of the art. J. Biosci. 48: 673-688.

References pertaining directly to the project are listed in the enclosed publication.