Project Report (June 2001 - May 2002)

Project Title: Insertion of Genetic Material for Oxalate Oxidase into Peanut for Improving Resistance to Sclerotinia Blight and Southern Stem Rot

Investigators:

Principal Investigators: E.A. Grabau and P.M. Phipps, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech

Other Laboratory Personnel: D.M. Livingstone (postdoctoral research associate) and J. Hampton (senior laboratory specialist).

Overivew of Project Results:

Goal: to regenerate transformed peanut plants expressing an oxalate oxidase gene for improved resistance to fungal disease.

Dr. Livingstone joined the project in October 2001 and has provided leadership in the tissue culture aspects of obtaining regenerable embryogenic cultures (REC) from commercial peanut cultivars. He has continued to advance our capabilities to initiate and maintain embryogenic peanut callus. He has optimized selection of transformed peanut cultures using hygromycin as the selectable marker. He has transformed peanut REC by particle bombardment with the gene gun and has selected for REC containing the active oxalate oxidase gene. He has also utilized another reporter gene (the gene for green fluorescent protein) as a method for following transformation and transgene expression that does not involve sacrificing plant material. Dr. Livingstone has recently obtained regenerated peanut plants from cultivar NC12C, 7 months after initiation of the cultures from peanut callus.

Ms. Hampton has been analyzing factors that contribute to pathogenicity of the fungus, *Sclerotinia minor*. This will provide us with the ability to intervene in the infection process with appropriate antifungal measures to improve resistance.

Future plans:

We will assay transformed REC to monitor the extent of transformation obtained. Once satisfied that the entire plant is transformed (not a mixture of transformed and non-transformed material), we will regenerate peanut plants expressing the oxalate oxidase gene. We anticipate the first transformed plants by the end of the year. Stable transformation will be confirmed and transformed plants and their progeny tested for disease resistance.

Funds expended:

As of May 29, 2002, we have spent $70,266 on materials and supplies, salaries and benefits for postdoctoral research associate and senior laboratory specialist.

Respectfully submitted, May 29, 2002:

Elizabeth A. Grabau

EAG for P.M. Phipps

Elizabeth A. Grabau

Patrick M. Phipps

TECHNICAL DETAILS TO ACCOMPANY PROJECT REPORT

Introduction
Peanut embryos can be induced to form a variety of callus types in tissue culture by including growth substances such as picloram or 2,4 D in the culture medium. One of these callus types is regenerable. Regenerable embryogenic callus (REC) can be derived from zygotic embryos of mature dry seed or zygotic embryos of immature seed. Although immature seed is available in limited supplies and subject to higher rates of contamination than mature seed, callus derived from it is more regenerable. Peanut can be genetically transformed by microprojectile bombardment into REC followed by a period on a selective medium. Typically, hygromycin B is the preferred selective agent.

Initiation and Maintenance of Embryogenic Callus
We have successfully initiated REC derived from mature dry seed of the cultivars NC-7, Wilson, Perry, NC12C, VA 98R and VA 92R. We have also obtained REC derived from immature embryos of the cultivars NC-7, Wilson and Perry. In general, the doubling time for REC is approximately one month.

Selection Parameters
The growth of non-transformed peanut REC (Fig. 1) is significantly restricted on medium containing 10 mgl^{-1} hygromycin B. 90% of callus is killed within 3 weeks on 20 mgl^{-1} hygromycin B. On 40 mgl^{-1}, 100% is dead within 3 weeks. We used 40 mg/l to select for transformed REC. Growth of transformed material is shown in Figure 2.

Transformation
We have bombarded REC from cultivars NC-7, Wilson, Perry, NC12C and VA 98R with genes coding for hygromycin resistance and oxalate oxidase and these are currently on selective medium (MS salts and vitamins, 3% sucrose, 1 mgl^{-1} glutamine, 3mgl^{-1} picloram supplemented with 40 mgl^{-1} hygromycin B).

Four months after bombarded callus was placed on the selective medium, we have 189 lines of transformed NC-7, 160 lines of transformed Wilson, and 69 lines of transformed Perry. NC12C and VA 98R callus has not been on the selective medium long enough to estimate the number of transformed lines. Callus cultures derived from immature embryos of the cultivars NC-7, Wilson and Perry have also been bombarded and are now being screened on the selective medium.

Reporter Gene Assays
Although hygromycin B is an effective selective agent for transformed peanut tissue, survival of adjacent untransformed tissue can occur (Fig. 4). This means that it is important to maintain transformed tissue for up to 6 months on selection to ensure high rates of germline transformation (to obtain non-chimeric tissue prior to regeneration). Green fluorescent protein (gfp) is also being used as a non-destructive reporter gene assay (Fig. 5).

In addition to its role as a putative disease resistance gene, oxalate oxidase can also be used as a reporter of transgene activity. We have optimized two assays for oxalate oxidase activity, one a histochemical stain to visualize transformed sectors (Fig. 3) and the other a quantitative assay to measure activity in various transgenic lines (Fig. 6).

Regeneration
Somatic embryos from REC (derived from mature seed) have been regenerated in tissue culture at a frequency of 5%. Regeneration rates for REC derived from immature seed are expected to be higher. Figure 4 illustrates a regenerated plant from NC12C.

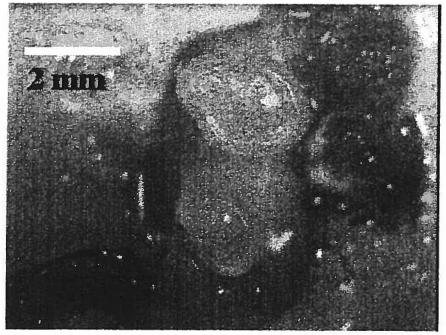

Fig. 1: Callus prior to selection

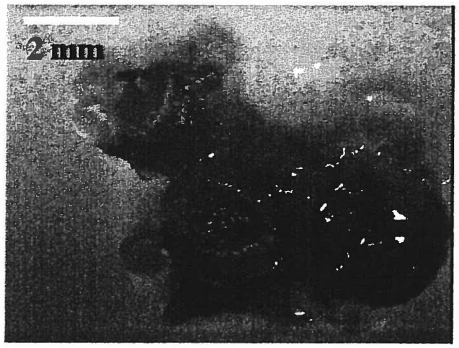

Fig.2: Bombarded callus after 3 weeks on 40 mgl^{-1} hygromycin B. Small partly transformed embryos are growing out of non-resistant tissue.

Fig. 3: Callus after 2 months on 40 mgl^{-1} hygromycin B. A histochemical assay shows oxalate oxidase activity (dark purple stain).

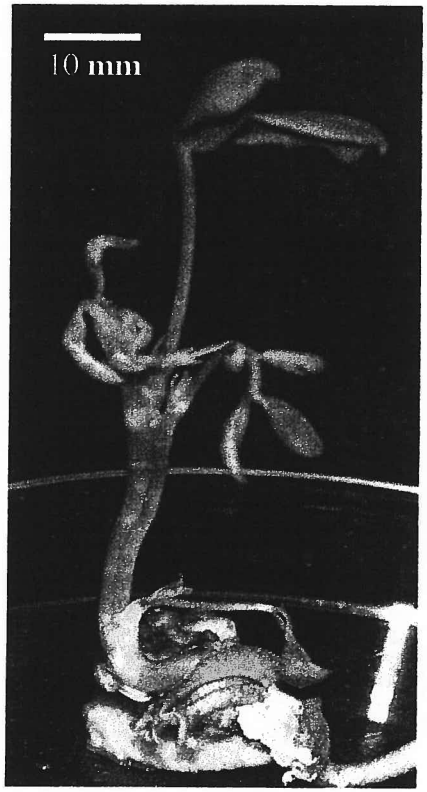

Fig.4: Regenerating peanut plant 7 months after initiation of callus [derived from mature seed (cv. NC12C)].

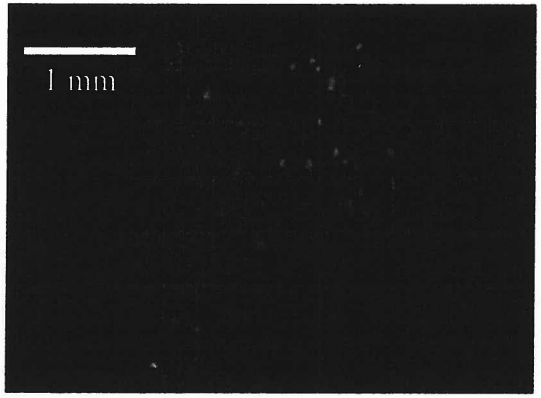

Fig.5: g*fp* (green fluorescent protein) expression in REC 3 weeks after transfer to selective medium. There are many transformed cells in close proximity.

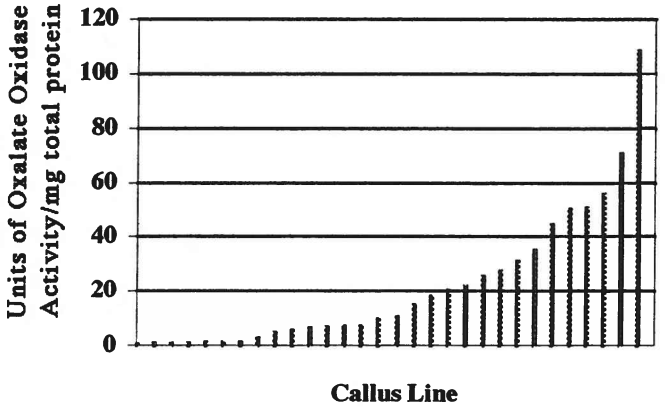

Fig. 6: Oxalate oxidase activity in transformed callus lines (cv. NC-7) showing a large variation in levels of transgene activity.

Virginia Tech

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May 29, 2002

Mr. Russell Schools
Virginia Peanut Growers Association, Inc.
23020 Main Street
P.O. Box 356
Capron, VA 23829-0356

Dear Mr. Schools:

Enclosed with this letter please find an annual report for project #437751, directed by E.A. Grabau and P.M. Phipps, covering the period from June 2001 through May 2002. I have limited the report to two pages plus photographs as requested. I would be happy to provide additional details if needed.

I'm sorry about any potential misunderstanding concerning submission of this report. In previous conversations with Sponsored Programs at Virginia Tech, I was informed that a final report was not due until 90 days after the project funding was completed. I've enclosed a copy of a letter that was forwarded to me last Friday from Sponsored Programs indicating that the project would continue until all the funds had been used. We will complete year 1 of the project within the next several months. Should I forward an updated progress report to you at that time?

Thank you for your ongoing support of this project. I'd like to express my continued enthusiasm for the great promise I feel this approach holds for improving the efficiency of peanut production. The skills that are being developed should allow Virginia Tech to become an important leader in peanut transformation. In addition, the products of this work are expected to benefit the peanut industry through a reduction in fungicide use and an increase in yield.

The results of this project will be presented at the annual APRES meetings in July. The titles of two abstracts are included below.

Genetic Transformation of Peanut for Resistance to *Sclerotinia minor*.
D.M. LIVINGSTONE*, J.L. HAMPTON, P.M. PHIPPS, E.A. GRABAU.

Growth and Oxalic Acid Production in Liquid Culture by Isolates of *Sclerotinia minor*. J.L. HAMPTON, D.M. LIVINGSTONE*, T. BOLUARTE-MEDINA, F. MEDINA-BOLIVAR, B.B. SHEW, J. HOLLOWELL, P.M. PHIPPS, E.A. GRABAU.

Sincerely,

Elizabeth A Grabau

Elizabeth A. Grabau
Associate Professor

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November 8, 2001

Sherry Whitaker
Contract and Grant Administrator
Office of Sponsored Programs
Collegiate Square, Box 209
460 Turner Street, Suite 306
Blacksburg, Va. 24060

Dear Ms. Whitaker:

This letter is to inform you that the three Research Projects funded by the National Peanut Board and handled by the Virginia Peanut Growers Association may continue past the contract dates, due to the lateness in getting the funding started. The projects and funding may continue until the approved amount for each project has been used.

The three projects are #437751 by E. A. Grabau, #437749 by Dr. C. W. Swann and #437750 by R. W. Mozingo.

Sincerely,

Russell C. Schools

Russell C. Schools
Executive Secretary

RCS/ntr
Cc: Walt Mozingo
Dr. Pat Phipps
Dr. Charles Swann