IBC 91-1

Registration Document for RECOMBINANT DNA EXPERIMENTS June 28, 1991

1. *Principal Investigator:* Gary M. Hellmann Ph.D

2. *Location:* Bowman Gray Technical Center, Reynolds Blvd., Winston-Salem, NC

3. *Laboratory:* BGTC 611-13E/011

4. *Title:* Molecular cloning of root-specific alkaloid biosynthesis genes from near-isogenic *Nicotiana tabacum* lines

5. *Will experiments be carried out in E. coli (X Yes; __No) or other prokaryotic host (X Yes; __No)*

6. *If yes, describe specific host, vector, DNA to be inserted, and nature of the experiment*

Tobacco cDNA inserted into vector pBluescript II KS+ and propagated in *E. coli* strain DH5a. Mobilization of cloned sequences into *Agrobacterium tumefaciens* strain LBA 4404 (see attached sheets)

7. *Physical containment* K-12; BL-1

8. *Will experiments be carried out in eukaryotic cells (X Yes; __No)?*

9. *If yes, describe specific host, vector, DNA to be inserted, and nature of experiment. If a viral vector is to be used, will infectious virus be generated?*

Introduction of tobacco root cDNA in binary transformation vector KYLX-71 into *N. tabacum* leaf discs or electroporation of *N. tabacum* protoplasts (see attached sheets)

10. *Physical containment:* BL-1P

11. *Will studies include attempts to obtain expression of a foreign gene, other than those used for selection purposes (X Yes; __No)? If yes, what proteins?*

Bacterial chloramphenicol acetyl transferase (CAT), bacterial beta glucuronidase (GUS) (see attached sheet)

12. *Other personnel associated with this project:*

Mr. Richard Reich, Mr. Ken Bridle, both of RJR

I acknowledge my responsibility for the conduct of this research in accordance with Section IV-B-5 of the NIH Guidelines and agree to comply voluntarily according to Section VI of the NIH Guidelines

Gary W. Hellmann — 919-741-0735 — 6/28/91
(Principal Investigator) (Telephone) (Date)

Experiment terminated: Gary W. Hellmann — 2/24/94
(Principal Investigator) (Date)

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This registration document has been approved by the RJR Institutional Biosafety Committee:

Joel A. Seckar — 3/11/94
(Chairman, RJR Biosafety Committee) (Date)

Project Abstract:

Biosynthesis of tobacco alkaloids (specifically nicotine, nornicotine, anatabine, anabasine) has been demonstrated to occur primarily or exclusively in root tissues. The alkaloids thus synthesized are subsequently transported to the aerial parts of the plant where they accumulate in cell vacuoles. Induction by hormones (release of apical dominance due to physical removal of the terminal growing tip) followed by a burst in the appearance of leaf alkaloids suggest that genes involved in alkaloid biosynthesis are transcriptionally regulated. Much is known concerning the intermediates in alkaloid biosynthesis, and a number of enzymatic activities have been characterized in crude or partially purified extracts. However, very little is known about the actual enzymes that regulate alkaloid biosynthesis and nothing concerning the physical structure of their corresponding genes. Genetic studies have revealed that two dominant "genes" contribute to nicotine accumulation in *N. tabacum*. These genes have been dubbed A and B, are inherited in an unlinked Mendelian fashion, and contribute independently to the sum total of nicotine accumulation. The relationship of these two "genes" to partially characterized enzymatic activities reported to participate in alkaloid biosynthesis is not known.

In the present series of experiments, cDNA libaries will be constructed from root tissues of four lines of tobacco which are near-isogenic with the exception of nicotine biosynthesis. The lines have been previously characterized genetically to possess AABB, AAbb, aaBB, and aabb genotypes. Total cDNA will be prepared from root tissue samples taken at a time point when a maximal difference in the nicotine accumulation levels of AABB and aabb lines are noted from mass spectrometric analysis of fresh tissue samples. Quality of mRNA templates will be ascertained by *in vitro* translation of mRNA preparations. In addition, 2-dimensional electrophoretic analysis of the [^{35}S]methionine-labeled translation products will be performed as a preliminary step towards characterizing root-specific protein differences between the lines. These libraries will be constructed in pBluescript II KS+ and introduced into *E. coli* strain DH5a by electroporation. All colonies will be harvested and propagated in 96-well microtiter plates. Duplicate filter sets of colony DNA will be screened with ^{32}P-labeled single-stranded cDNA probes synthesized from either AABB or aabb root mRNA. Colonies exhibiting exclusive signals from AABB probes will be selected as first round potential candidates for further analysis. Uniqueness of the clones will be assessed by cross-hybridization and then grouped into families. Finally, representative clones from each group will be utilized as probes against RNA isolated from all four tobacco lines. The number of clones displaying the desired characteristics through these screening rounds will be evaluated before the next procedure. If the number is suffiently small, limited sequence information will be obtained from the termini of the clones. If the clone appears to contain an entire coding region it will be transferred to the binary plant expression vector KYLX-71, a vector possessing an enhanced CaMV 35S promoter, a rbcS polyadenylation signal, and T-DNA borders. Plants of the aabb genotype will be transformed with each of the clones and examined for altered nicotine biosynthesis. In

the development of this system, it is anticipated that trial transformations (both *Agrobacterium*-mediated as well as via electroporation for transient expression) will be performed using binary vectors carrying a selectable marker (Kanamycin resistance), as well as the reporter genes CAT (chloramphenicol acetyltransferase) and GUS (B-glucuronidase).

Physical containment:

The proposed experiments require containment at Biosafety Level 1 (BL-1). The following procedures and protocols have been established to comply with this level of containment: Access to laboratory 611-13E/011 is limited to authorized personnel. This is indicated by signs posted at the entrances to the laboratory from the adjacent corridor indicating "Biohazard", "Radioisotope usage", and "No eating, drinking, smoking." The laboratory doors are locked when authorized laboratory personnel are not present.

All laboratory bench surfaces are impervious to liquids and are covered with disposable liquid-adsorbing, plastic backed toweling material. All contaminated liquids are decontaminated by the addition of a commercial germicide before disposal. All contaminated solids including discarded culture plates are placed in marked "biohazard" plastic bags and sterilized by autoclaving prior to disposal. All pipetting is done using mechanical or electrical pipetting devices.

All personnel have been instructed to wash hands after any procedures involving recombinant molecules. Four sinks are provided in the laboratory for hand washing. Laboratory coats and safety glasses are general requirements of all personnel who enter any laboratory at RJR.

An insect and rodent control program is in effect at RJR BGTC contracted through Wilson Pest Control. The equipment in the laboratory is less than 10 years old and is in good condition. The laboratory floors are cleaned nightly. Maintenance personnel have been instructed in standard laboratory safety precautions as well as being specifically informed as to the nature of the work in the laboratory. Windows in the laboratory are of the non-opening type, minimizing the possibility of insect contamination.

Transgenic tobacco will be propagated in growth chambers in laboratories which are locked when authorized personnel are absent. All transgenic materials will be completely inactivated by autoclaving before disposal.

Biological Containment:

All recombinant DNA experiments will involve the use of derivatives of *E. coli* K-12 which are not conjugation proficient. The strains to be used are JM83, JM101, JM109, TB1, HB101, DH5, XL-1 Blue, and DH10B.

For plant transformations, *Agrobacterium tumefaciens* strain LBA 4404 will be utilized. This strain has been disarmed for the production of plant growth regulators and supplies the trans acting *vir* functions necessary for the mobilization of the T-DNA region from binary vectors into plants. This commonly used strain is not considered a plant pathogen.