RICE UNIVERSITY

Metabolic Engineering of *Catharanthus roseus* Hairy Roots
Using an Inducible Promoter System

by

Erik H. Hughes

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

[Signatures and names]

Ka-Yiu San, Professor
Bioengineering and Chemical Engineering

Larry V. McIntire, E.D. Butcher Professor, Chair
Bioengineering and Chemical Engineering

Susan I. Gibson, Adjunct Associate Professor
Biochemistry and Cell Biology

Jacqueline V. Shanks, Adjunct Professor
Bioengineering

HOUSTON, TEXAS
APRIL, 2003
ABSTRACT

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Plant metabolic engineering is a developing field still in need of improved tools and an increased understanding of relevant pathways. This thesis addresses both those needs by testing a new tool for improved metabolic engineering studies and utilizing that tool for the exploration of monoterpenoid indole alkaloid biosynthetic pathways. Using GFP as a model protein in *Catharanthus roseus* hairy roots, we report that the glucocorticoid inducible promoter system is active and has a tightly controlled, reversible, and dosage-dependent response to dexamethasone. Furthermore, it provides an improved negative control useful for the study of genes affecting alkaloid synthesis.

The most exhaustive transgenic studies reported here focus on the indole pathway. Expressing a feedback-resistant *Arabidopsis* anthranilate synthase \( \alpha \) subunit results in dramatic increases in tryptophan and tryptamine yields. On induction, tryptophan increases from undetectable levels to 2.5 mg/g DW, while tryptamine increases from 25 \( \mu g/g \) DW to 267 \( \mu g/g \) DW. Additionally, a transient improvement in lochnericine yield indicates a possible increase in alkaloid flux countered by tight regulation of alkaloid levels. In lines transgenic for inducible tryptophan decarboxylase, serpentine specific
yields increased by as much as 129% on induction. The reported studies on the indole pathway demonstrate successful methods to improve indole flux and show that increased indole flux can lead to improvements in certain alkaloids.

Precursors from the complementing terpenoid pathway are also required for alkaloid synthesis. A feeding study utilizing an intermediate and a specific inhibitor of the nonmevalonate pathway validates the importance of this pathway for improved alkaloid yields and points to the potential success of upstream metabolic engineering efforts. In preliminary results, improved yields of certain alkaloids are reported for hairy root lines transgenic for 1-deoxy-D-xylulose-5-phosphate synthase and geraniol 10-hydroxylase, while an ORCA3 study highlights some potential problems with the use of transcriptional activators. In the last study, we focus on the engineering of a valuable alkaloid pathway and report a transgenic hairy root line overexpressing the full coding sequence of tabersonine 16-hydroxylase. On induction, the line produces 16-methoxytabersonine. Overall, a valuable new tool is introduced and subsequently used to manipulate the indole, terpenoid, and alkaloid pathways.
Acknowledgements

A number of individuals deserve special thanks for the contributions that they have made to my Ph.D. experience. Funding from NSF grants (BES-0003730, BES-9906978) and a NIH training grant (T32-GM08362) is also acknowledged. I remain grateful to

Dr. Ka-Yiu San for providing unmatched support and enthusiasm as the greatest advisor a Ph.D. candidate could hope to have;

Dr. Jacqueline Shanks, Dr. Rajiv Bhadra, and Dr. John Morgan for providing helpful guidance and laying the groundwork upon which my work was built;

Dr. Sue Gibson and Dr. Dennis Hong for patiently converting an engineer into a plant molecular biologist;

Dr. Larry McIntire, Dr. San, Dr. Shanks, and Dr. Gibson for both teaching me in the classroom and serving on my thesis committee;

Arnez and Araceli for happily providing help to me among the many other graduate students who depend on them;

Mary Harrison for letting me borrow everything and most importantly showing me where it was hidden;

Classmates and Labmates (Tina, Susana, Ailen, Henry, Ravi, Aasheesh, Kelly, Murali, Ganesh, Donna, Carrie, Daniel, John, Miles, Emily, Roy, Stacy, Brey, and Dave) for providing help sometimes and more often needed distraction;

Friends from Rice and beyond the parking gates for making the significant and often unrewarded effort of research bearable;

My parents for supporting my choices;

And Sarah for giving me everything that really matters.
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Chapter 1

Introduction

This chapter begins with a background section that discusses the importance of plant alkaloids and the promise of metabolic engineering for increasing their yields. The subsequent section highlights specific factors which control alkaloid biosynthesis and related problems that plant metabolic engineering must overcome. This is followed by an overview of previous attempts to metabolically engineer \textit{C. roseus} indole alkaloid pathways and a discussion of the current availability of genes. Finally, the chapter concludes with a project overview section that states the general goals of this research and a thesis outline that summarizes the chapters. Significant sections of this chapter were published as a review article (Hughes & Shanks 2002).

1.1 The Study of Plant Alkaloids

Alkaloids are a structurally diverse class of nitrogenous compounds that are found in many plants and often exhibit physiological activity. Throughout history, plants that produce alkaloids and their extracts have been exploited for their medicinal and toxic properties. Modern examples of widely used plant-derived alkaloids include analgesics (morphine and codeine), stimulants (caffeine and nicotine), and chemotherapeutics (vincristine, vinblastine, camptothecin derivatives, and paclitaxel). In plants alone, over 12,000 alkaloid structures have already been elucidated (Wink 1999) providing drug companies with a diverse set of structures valuable for pharmacological screening (Shu 1998; Verpoorte 2000; Verpoorte et al. 2000). Such complexity, however, has made
elucidation at the metabolite and enzyme level difficult. In 1995, Kutchan estimated that only 80 enzymes involved in the synthesis of various classes of alkaloids had been discovered and partially characterized (Kutchan 1995). Although still limited, our understanding of alkaloid synthesis has improved due to a focus on particular model plants and specific classes of alkaloids. One such model organism has been *Catharanthus roseus* which produces a wide variety of terpenoid indole alkaloids including the anti-hypertension agents ajmalicine and serpentine and the chemotherapeutics vincristine and vinblastine.

As is common for secondary metabolites produced by plants, alkaloid yields are often very low necessitating expensive recovery techniques. For example, yields of vincristine and vinblastine have been reported to be 1 g and 20 mg per 1000 kg of plant material, respectively (Tyler 1988). Metabolic engineering through the directed modification of metabolic pathways by recombinant DNA technology offers great promise in increasing yields but requires a thorough understanding of the biosynthetic pathways that is often lacking (Bailey 1991; Stephanopoulos 1994). Fortunately, certain advancements including the development of plant cell and tissue cultures as laboratory systems have expedited the elucidation of pathways at the metabolite and enzyme level. While such systems have proved scientifically valuable, they are not yet plausible production systems. It was initially believed that culture systems could act as a stable supply immune to the environmental fluctuations, habitat limitations, and slow growth rates of field plants, but the strict native regulation of alkaloid synthesis has often resulted in low or nonexistent levels of the desired alkaloids. High overall productivity of
alkaloids has only rarely been achieved as with berberine production in *Coptis* cells and paclitaxel production in *Taxus* cell cultures (Ketchum et al 1999; Kutchan 1995; Verpoorte et al 1999; Verpoorte et al 1991; Zenk et al 1988). Most of the efforts, however, have not focused on genetically engineered cell cultures, tissue cultures, or plants but rather on non-engineered cell lines studied under elicitation, with precursor feeding, or in optimized media. These classical methods have certainly hastened characterization efforts, but metabolic engineering offers the most promising method for improved product composition and increased alkaloid yield of plants and cultured cell systems. In proceeding though, scientists must be careful to recognize the unique problems in engineering plant metabolism and overcoming native regulation.

1.2 Considerations for Plant Metabolic Engineering

The primary difficulty encountered so far in the production of alkaloids has been the tight and largely unexplored regulation of secondary metabolism. Cell cultures have been incapable of synthesizing a number of commercially important alkaloids including morphine, codeine, scopolamine, vincristine, and vinblastine (Verpoorte et al 1999; Verpoorte et al 2000). In *A. belladonna* and *C. roseus*, a number of studies have demonstrated that developmental stage, environmental influence, cellular compartmentation, and tissue differentiation exert significant control over alkaloid accumulation (DeLuca & Cutler 1987; DeLuca & St.-Pierre 2000; DeLuca et al 1998; Hashimoto & Yamada 1994; Meijer et al 1993b; St.-Pierre et al 1999).
Alkaloid production in *A. belladonna* and *C. roseus* is developmentally regulated with the highest levels occurring in younger, faster dividing regions of the plant (DeLuca & St.-Pierre 2000). Furthermore, some steps require environmental stimuli. For example, light proves necessary for the synthesis of vindoline, an important precursor of vincristine and vinblastine in *C. roseus* (DeLuca et al 1989a; DeLuca & St.-Pierre 2000). The division of higher plant cells into organelles also presents challenges not encountered in bacteria. The pathways of interest often involve multiple compartments resulting in transport limitations and sequestered pools of metabolites. As an example, the synthesis of strictosidine, the precursor to the indole alkaloids, requires three cellular compartments. Tryptophan and the terpenoid precursor geraniol are synthesized in the plastids, tryptophan is then decarboxylated in the cytosol, and the two moieties are condensed in the vacuole (DeLuca & St.-Pierre 2000). This compartmentation is particularly important to consider as incorrect targeting of transgenes will leave them without the necessary substrates (Herminghaus et al 1996).

The lack of differentiation in cell cultures has also been a barrier to successful alkaloid production. *C. roseus* hairy roots, a fast growing, differentiated tissue culture generated by *Agrobacterium rhizogenes* infection, are known to accumulate far higher levels of alkaloids than undifferentiated cell and callus cultures (Moreno-Valenzuela et al 1998). The biosynthesis of vindoline, however, only exists at significant levels in shooty teratomas (O’Keefe et al 1997) or shoots regenerated from callus (Miura et al 1988). Furthermore, it has also been shown that two late enzymes required for vindoline synthesis show activity only in particular specialized cells of the stem and leaves (St.-
Pierre et al 1999). Clearly, alkaloid biosynthesis is regulated at a number of levels and plant cells present several challenges to the manipulation of these pathways.

Metabolic engineering is currently able to meet some of the challenges and offers the greatest hope for overcoming native regulatory controls. However, some remaining problems are not easily overcome. There remains a great deal to be learned about the regulation of alkaloid pathways, and many important genes still have not been cloned. Low levels of enzymes and metabolites related to alkaloid pathways have made pathway elucidation, enzyme purification, enzyme characterization, and cloning difficult. The late stages of secondary metabolite pathways are often species-specific, making model plant studies and sequencing efforts like the Arabidopsis project of limited use. Additionally, cofactor limitations, the importance of catabolism, feedback controls, and the influence of subcellular and tissue transport are poorly understood. For the generation of transgenics, some problems also still remain. Clone generation and characterization is often a time-consuming process particularly complicated in species that remain difficult to transform and regenerate. The stability of gene expression is also a concern as cell cultures are often unstable and cosuppression of transgenes is sometimes encountered.

Even given these difficulties, there have been a number of metabolic engineering successes in manipulating plant metabolism, and the opportunities will continually expand as characterization and cloning efforts progress. The tools for genetic transformation including Agrobacterium and biolistic approaches have undergone significant development, and continued gains will be made on the introduction and controlled expression of multiple genes. As the field progresses, the expression of
enzymes with advantageous properties, the blocking of competitive pathways or catabolism, the production of new alkaloids in alternative plant species, and the overexpression of important endogenous pathway genes or transcription factors will become possible (Verpoorte et al 1999; Verpoorte et al 2000).

1.3 Metabolic Engineering of C. roseus Alkaloid Pathways

Perhaps the best way to illustrate the problems and successes of plant metabolic engineering for the production of alkaloids is to focus on C. roseus, the model organism studied in this thesis. As the enzymology and pathway elucidation for alkaloids in general (Facchini 2001; Hashimoto & Yamada 1994; Kutchan 1995; Roberts & Strack 1999) and the indole alkaloids (DeLuca & St.-Pierre 2000; Meijer et al 1993b; Misra et al 1996) has been reviewed elsewhere, the focus here will be placed on metabolic engineering efforts.

The terpenoid indole alkaloids (TIAs) produced in C. roseus have been extensively studied due to their pharmaceutical importance but have presented a number of challenges. While hairy root and cell cultures produce a number of TIAs, they do not produce the valuable dimeric alkaloids, vinblastine and vincristine. A number of the reasons for this inactivity have already been noted. It is also a challenging species with which to work as no suitable regeneration protocol has been reported leaving cell and tissue cultures as the only available systems for metabolic engineering. Other difficulties that are more characteristic of plant systems in general have also been encountered. Recently, the metabolic map to the TIAs had to be revised when NMR confirmed that the
newly discovered nonmevalonate terpenoid pathway was the primary source of isopentenyl diphosphate for secologanin synthesis (Contin et al. 1998; Lichtenthaler 1999). Determining the function of cloned genes has also been difficult as shown by the recent functional confirmation of secologanin synthase (SLS) from a cDNA that was isolated in 1992 (Irmler et al. 2000). Overall, however, the lengthy cloning efforts focused on *C. roseus* have been very successful. As shown in Figure 1-1, currently cloned genes involved in monoterpenoid indole alkaloid synthesis include those required for the synthesis of terpenoid precursors, indole precursors, and various specific alkaloids.

The first preliminary step in approaching the engineering of the pathways in *C. roseus* has been an attempt to quantify the relative importance of the terpenoid and indole pathways with precursor feedings. The basis of such experiments is that high incorporation of a precursor into a target compound indicates that the precursor supply is limiting. Although the terpenoid pathway has generally been found to be limiting in hairy root and cell cultures (Morgan & Shanks 2000), a few cell lines have responded to indole feeding (Whitmer et al. 1998). Results in hairy roots further demonstrate that growth stage plays a key role in determining the relative importance of the two pathways (Morgan & Shanks 2000). Potentially important enzymatic steps have also been identified by correlating enzyme activity or gene transcription with alkaloid accumulation. For instance, representative studies on genes used in transgenic experiments have demonstrated that TDC activity coincides with alkaloid accumulation, while STR activity is relatively stable (Meijer et al. 1993b). While not definitive,
**Figure 1-1.** Cloned genes in the terpenoid, indole, and alkaloid pathways of *C. roseus*.

**DXS**, 1-deoxy-D-xylulose 5-phosphate synthase (Chahed et al. 2000); **DXR**, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Veau et al. 2000); **MECS**, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (Veau et al. 2000); **CPR**, cytochrome P-450 reductase (Meijer et al. 1993a); **G10H**, geraniol 10-hydroxylase (Collu et al. 2001); **10HGO**, 10-hydroxygeraniol NADP⁺ oxidoreductase (Teoh 2001); **SLS**, secologanin synthase (Irmler et al. 2000); **ASα**, antranilate synthase alpha subunit (Genbank accession AJ250008); **TDC**, tryptophan decarboxylase (DeLuca et al. 1989b); **STR**, strictosidine synthase (McKnight et al. 1990); **SGD**, strictosidine β-D-glucosidase (Geerlings et al. 2000); **T16H**, tabersonine 16-hydroxylase (Schroder et al. 1999); **D4H**, desacetoxyvindoline 4-hydroxylase (Vazquez-Flota et al. 1997); **DAT**, deacetylvinlindoline acetyltransferase (St.-Pierre et al. 1998).
indications from precursor and correlation studies provide valuable guidance for metabolic engineering experiments.

As STR and TDC were among the first genes cloned, they have also been the first employed in metabolic engineering studies. Constitutive expression of STR in cell cultures showed a surprising positive correlation to alkaloid levels (Canel et al 1998), but TDC expression resulted only in elevated tryptamine levels in transgenic cell cultures (Canel et al 1998) and crown gall calluses (Goddijn et al 1995). Although elevated levels of TDC did not result in improved TIA accumulation, an antisense clone showed that TIAs were absent when TDC activity was ablated (Goddijn et al 1995). From the results of feeding studies, it should not be surprising that TDC overexpression alone was ineffective. However, it should be noted that the presence of elevated indole precursor levels in cell cultures does appear to play a role in increased alkaloid levels after initial terpenoid feeding (Whitmer et al 2002a; Whitmer et al 2002b). TDC expression could therefore be valuable in concert with elevated terpenoid pools. While its individual expression effected little change, TDC has proved useful in other systems as a means of diverting flux to a metabolic sink to reduce undesired products in canola (Chavadej et al 1994) and manipulating the alkaloid contents of Cinchona officinalis (Geerlings et al 1999) and Peganum harmala (Berlin et al 1993). Single gene studies are unlikely to yield significant gains due to the tight regulatory networks, but they are important first steps in understanding and improving C. roseus alkaloid biosynthesis.

Another appealing strategy given that the indole alkaloid pathway involves over 20 steps is to rely on transcriptional regulators that activate many genes in the pathway.
In *C. roseus*, methyl jasmonate is known to be a strong elicitor of TIA production, and a jasmonate responsive regulator was recently cloned (van der Fits & Memelink 2000). Constitutive expression of the isolated regulator, ORCA3, resulted in induced levels of a number of genes including DXS, AS, TDC, STR, and D4H. Unfortunately, a few genes including G10H, which is necessary for secologanin synthesis, showed no induction. Although the cell line studied did not synthesize TIAs due to the terpenoid deficiency, loganin feeding resulted in a three-fold improvement in TIAs over control cultures. Since there are multiple controlled steps and the engineering of more than a few steps in long pathways is currently beyond our capabilities, transcriptional activators may provide one of the most powerful tools to modulate long and complicated pathways. One caveat, however, is that activators could elicit the expression of other pathways that are detrimental to cell growth and overall alkaloid yields.

A significant number of genes are now available for the metabolic engineering of TIA pathways in *C. roseus*, but reported efforts to date include only constitutive expression of TDC, STR, and ORCA3. A few of the available genes represent potentially important regulatory points and therefore continued engineering efforts are warranted. As the exact roles of available genes in increased alkaloid synthesis remain difficult to characterize, the best approach may be to simply generate transgenic lines using available genes that have shown promise in enzymatic and precursor feedings. Although such intervention may result in limited success due to substrate limitations, the activities of other enzymes, or feedback loops, such studies will clarify regulatory architecture. For instance, overexpression of an early branch point enzyme may point to
later limitations in flux that can be studied and addressed. Pursuing progress along these lines should result in an iterative process of improvement that will certainly increase our understanding of alkaloid biosynthesis and lead to improved future engineering efforts.

1.4 Thesis Overview

The focus of this thesis is the expansion of the reported metabolic engineering efforts in *C. roseus* driven by an inducible promoter allowing for improved analysis of transgenic studies. Within these studies, we use *C. roseus* hairy roots because they show substantially higher specific alkaloid yields in addition to improved biochemical and genetic stability over cell culture (Baiza et al 1999; Deus-Neumann & Zenk 1984; Moreno-Valenzuela et al 1998; Shanks & Morgan 1999). Initially, the activity of the glucocorticoid inducible promoter and our ability to generate transgenic hairy roots is tested with green fluorescent protein (GFP) as a model protein. As alkaloid yields vary substantially on a clonal basis and insertion of transgenes often results in varied activities and phenotypes (Bhadra et al 1993; Leech et al 1998), an inducible promoter offers an improved negative control over the previously reported constitutive expression studies by utilizing the same line as the control. Additionally, it also offers improved experimental flexibility by allowing for quantitative and temporal control. With the activity of the inducible promoter tested, the ability to engineer the indole pathway and its effect on alkaloid accumulation is studied. Previous precursor feeding work in our system has shown that alkaloid synthesis improved upon tryptophan feeding in the late exponential stage of growth (Morgan & Shanks 2000). This knowledge combined with the extensive
regulatory and enzymatic characterization of the indole pathway motivated and enabled our engineering efforts. In addition to the engineering of this pathway, our overall goal remained an improved understanding of methods to successfully engineer alkaloid production. As manipulation of the terpenoid pathway will likely be necessary for substantial alkaloid gains, an initial feeding study was done to determine the importance of the non-mevalonate terpenoid pathway and the likelihood of successful metabolic engineering efforts. Recognizing the necessity of understanding the roles of a number of genes and pathways, preliminary efforts at utilizing our advantageous promoter system for the engineering of a signaling pathway (ORCA3), the terpenoid pathway (G10H, DXS), and a specific alkaloid pathway (T16H) were also undertaken. Overall, the thesis presents a valuable new tool for the engineering of alkaloid pathways, an improved understanding of the roles of a few relevant alkaloid pathways, and a number of new plant metabolic engineering studies.

1.5 Thesis Outline

Chapter 2 describes in detail the materials and methods used to conduct the research presented in this thesis. Methods specific to particular chapters or those that are slightly altered for a designated purpose can be found in the chapter for which they were used.

Chapter 3 investigates the ability to generate transgenic hairy roots carrying a glucocorticoid inducible promoter and the activity of that promoter in C. roseus hairy
roots. The behavior of the inducible promoter is characterized within our system using GFP as the model protein.

Chapter 4 is focused on the use of the inducible promoter to drive expression of a feedback-resistant anthranilate synthase alpha subunit (AS\(\alpha\)) from Arabidopsis. The chapter explores the metabolic effects caused by inducible expression of this gene. Specifically, it looks at the effects on tryptophan, tryptamine, and the indole alkaloids during two growth stages.

Chapter 5 explores similar metabolic effects from indole pathway engineering but on clones engineered for inducible expression of tryptophan decarboxylase alone or coupled with the feedback-resistant AS\(\alpha\).

Chapter 6 investigates the role of the non-mevalonate terpenoid pathway in supplying precursors for increased alkaloid synthesis. In particular, it examines the role of the first two genes of the non-mevalonate pathway and attempts to evaluate the potential outcome of metabolic engineering of these steps through a precursor and inhibitor study.

Chapter 7 presents preliminary data regarding the generation of clones transgenic for inducible DXS, G10H, and ORCA3 and the effects of inducible expression on alkaloid synthesis.

Chapter 8 reports the cloning of the 5’ end of the tabersonine 16-hydroxylase gene and expression of the full coding sequence behind the inducible promoter in transgenic hairy roots. Preliminary data regarding metabolic effects on induction are also reported.
Chapter 9 summarizes the advances reported in this thesis and is followed by two appendices. Appendix A presents a genetic sequence highly homologous to T16H that was cloned by chance. Appendix B reports some initial data on measuring total protein levels within our system.
Chapter 2

Materials and Methods

The following thesis uses a variety of methods from the fields of microbiology and molecular biology. As a wide number of sources document these techniques, they will not be covered here unless vital to the work presented. Current Protocols in Molecular Biology among other extensive sources can be used as a reference for such basic techniques as running TAE agarose gels, plasmid ligation, PCR, and restriction enzyme analysis. Microbiological techniques for *Escherichia coli* including the preparation of media and competent cells are also extensively detailed elsewhere. Therefore, the primary focus here will be placed on techniques unique to the hairy root work.

2.1 Microbiology

2.1.1 *Agrobacterium* Media

*Agrobacterium rhizogenes* ATCC 15834 was grown in YEM media containing 10 g/L mannitol, 0.2 g/L NaCl, 0.2 g/L MgSO₄•7H₂O, 1 g/L yeast extract, 0.004 g/L FeCl₃, and 0.66 g/L K₂HPO₄•3H₂O. Appropriate antibiotics were added as needed (50 mg/L kanamycin), and 15 g/L agar was added to make solid plates. For the purpose of infection, liquid cultures were grown for 36 hours at 28 °C and 200 rpm.
2.1.2 Electrocompetent Agrobacterium and Electrotansformation

*Agrobacterium* remains difficult to transform, and the following protocol was used to prepare electrocompetent cells. To start the culture, inoculate 100 mL YEM media with 1 mL of overnight *A. rhizogenes* culture. Grow the cells at 28 °C with shaking to an ABS600 of 0.5 to 1.0. Chill the growth flask on ice for 20 minutes and centrifuge in a cold rotor at 4000g for 10 minutes. Remove the supernatant and resuspend in 20 mL of 10 mM HEPES (pH 7). Centrifuge again. Resuspend in 10 mL of 10 mM HEPES (pH 7). Centrifuge again and resuspend in 2 mL of cold 10% glycerol/10 mM HEPES (pH 7). Transfer to microfuge tubes and centrifuge again. Repeat the resuspension in 2 mL 10% glycerol/10 mM HEPES (pH 7) twice with centrifugation. Resuspend to a final volume of 300 μL in cold 10% glycerol/10 mM HEPES (pH 7). If arcing occurs with these cells, continue washing. Otherwise, put 40 μL aliquots in a -80 °C freezer for storage. For electrotansformation, gently thaw the cells on ice. Mix 40 μL of the cell suspension with 2 μL DNA and let sit on ice for 1 minute. Set the pulsing apparatus to 25 μF, 2.5 kV, and 200 Ohms. Transfer the suspension to a cold electroporation cuvette and shake to the bottom. Pulse once and immediately resuspend cells in 0.5 mL YEM. Incubate for 3 hours at 28 °C and 200 RPM. Spread on selection media plates and wait approximately 2 days for colonies.

2.1.3 Glycerol Stocks

All glycerol stocks from *E. coli* and *A. rhizogenes* strains were numbered and stored at -80 °C with appropriate documentation in a glycerol stock reference notebook.
Liquid cultures of selected colonies were spun down in a microcentrifuge, and the pellets resuspended in a 20% glycerol 10 mM HEPES (pH 7) solution. They were then immediately frozen at −80 °C. Liquid cultures were inoculated by stabbing the solid glycerol stock with a sterile toothpick.

2.2 Molecular Biology

2.2.1 Basics

Restriction and other commonly used enzymes were purchased from Promega or New England Biolabs except Sdal (Sse 83871) which is uncommon and was purchased from MBI Fermentas. Chemically competent *E. coli* cells were prepared from DH10B cultures following a laboratory protocol or alternatively purchased from Invitrogen (TOP10F’ cells). Common plasmids used included pBluescript II KS (Stratagene) and the pTA7002 glucocorticoid inducible promoter (Figure 2-1) generously provided by Dr. Nam-Hai Chua. Another useful plasmid was pCR2.1-TOPO (Invitrogen) which is part of a kit that allows the direct cloning of PCR products synthesized with Taq polymerase. Ligation was performed with gel-purified fragments prepared using the Qiagen QIAEX II kit. Plant DNA was prepared from hairy roots or seedlings using the Sigma GenElute Plant Genomic DNA Miniprep kit, and *E. coli* plasmid stocks were prepared using the Sigma GenElute Plasmid Miniprep kit.
Figure 2-1. Map of T-DNA segment from pTA7002. GVG is the constitutively expressed transcription factor. HPT allows for hygromycin selection of transgenic hairy root lines. The XhoI/SpeI cloning site contains the inducible gene of interest. For double gene constructs, the Sdal site can be used.
2.2.2 RNA and cDNA

In preparing RNA, a clean area inside a fume hood was utilized and all pipet tips and centrifuge tubes were RNase-free. Plant RNA was prepared using Invitrogen's Trizol according to instructions. Tissue was harvested and briefly blotted. Approximately, 0.3 mg of tissue was placed in a mortar with liquid nitrogen and ground to a fine powder. Before any thawing, 3 mL Trizol was added with subsequent homogenization and the mixture split equally into 3 microfuge tubes. While other samples were being prepared, these tubes were kept in dry ice. The following protocol was then followed. Incubate tubes at room temperature for 5 minutes. Spin at 12000g for 15 minutes at 4 °C. Transfer supernatants to new tubes. Add 0.2 mL chloroform and shake for 15 seconds. Incubate at room temperature for another 5 minutes followed by centrifugation as previously described. Transfer supernatants to fresh tubes and add 0.5 mL isopropanol. After shaking, leave at room temperature for 15 minutes and then spin at 12000g for 10 minutes at 4 °C. Discard supernatant and wash pellet with 75% ethanol (DEPC water). Mix gently and respin at 7500g for 5 minutes at 4 °C. Remove supernatant, spin briefly, and pipet away remaining liquid. Resuspend RNA in 20 μL DEPC-treated water with brief heating to 60 °C if necessary. Preparations from the same tissue can be combined at this point. Spin tubes at maximum speed in a microfuge for 5 minutes at 4 °C and transfer supernatants to new tubes. Store at −80 °C. If needed, cDNA was synthesized with M-MLV reverse transcriptase according to manufacturer's directions (Promega). Genes were then cloned from this cDNA preparation if introns
interrupted the coding sequence or alternatively from plant genomic DNA if no introns were present.

2.2.3 Plasmid preparation

In addition to the Sigma GenElute plasmid miniprep kit previously noted, two other protocols for plasmid preparation were utilized. The first is particularly useful when a large number of E. coli colonies carrying high copy plasmids need to be screened (Berghammer & Auer 1993). Approximately 2 mL of bacterial culture is spun at maximum speed in a microfuge for 30 seconds. The supernatant is removed and the pellet resuspended in 50 µL of lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 15% w/v sucrose, 2 mg/mL lysozyme, 0.2 mg/mL RNAse A, 0.1 mg/ml BSA). Let tube sit or shake for 5 minutes. Boil for 1 minute and put on ice for 1 minute. Centrifuge at maximum speed for 15-20 minutes in a microfuge. 8 µL of the supernatant can then be used directly for restriction enzyme analysis.

For A. rhizogenes cultures or cleaner E. coli plasmid preparations, another protocol is suitable. Spin down approximately 4 mL of culture and discard supernatant. For A. rhizogenes, resuspend pellet in 0.5 mL wash solution (0.5 M NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA), recentrifuge, and discard supernatant. After washing or directly in the case of E. coli, resuspend in 100 µL glucose/EDTA solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8)) with vortexing. Add 200 µL 0.2 N NaOH/ 1% SDS solution and vortex lightly. Incubate at room temperature for 5 minutes. Add 150 µL cold potassium acetate (3 M potassium, 5 M acetate, pH 4.8). Vortex gently
and store on ice for 5 minutes. Centrifuge for 15 minutes and move supernatant to new tube. Add 2 volumes EtOH, precipitate at -20 °C, and allow pellet to dry. Dissolve in 50 μL TE (10 mM Tris-HCl (pH 8), 1 mM EDTA, 20 μg/mL RNase A) and put in 37 °C bath for 30 minutes. At this point, E. coli plasmid preparations are suitable for restriction enzyme analysis or sequencing. A. rhizogenes preparations require further steps and concentration. Add 50 μL TE and 8 μL 3 M NaOAc (pH 5.2). To this mixture, add an equal volume chloroform/phenol. Vortex, spin 2 minutes, and move top phase to a new tube. Add equal volume 24:1 chloroform/isoamyl alcohol to top phase and repeat vortexing and spinning. Remove top phase to a new tube containing 2 volumes EtOH. Mix gently and precipitate at -20 °C for a few hours or -80 °C for 30 minutes. Spin at 4 °C for 10 minutes at maximum speed. Wash pellet with 70% EtOH and allow pellet to dry. Redissolve in 5 μL and use directly for restriction enzyme analysis of A. rhizogenes plasmid. The yields of A. rhizogenes plasmid are very low and bands below 1 kb will be hard to visualize in agarose gels.

2.2.4 Tail PCR

Tail PCR is used to permit the PCR based amplification of unknown sequences adjacent to known sequences. Within this thesis, it proved useful for the cloning of tabersonine 16-hydroxylase’s 5’ end. This protocol is taken from a literature source (Liu et al 1995). Three random primers (AD1 NTCGASTWTSGWGT; AD2 NGTCGASWGANAWGAA; AD3 WGTGAGWANCANAGA; S=C, G; W=A,T; N=A, C, G, T) are used. Three nested primers are then designed within the known
sequence. The TAIL1 primer is the one farthest from the sequence of interest, and TAIL3 is the closest. PCR reactions are then performed as follows. A primary reaction is initially performed (10.8 µL water, 2 µL Taq Buffer, 2 µL dNTP mix, 1 µL DNA preparation, 2 µL 2 µM TAIL1, 2 µL random primer (AD1, AD2, or AD3), 0.2 µL Taq).

The program for the primary reaction is as follows (1. 95 C 3’; 2. 94 C 30”; 3. 62 C 30”; 4. 72 C 1’30”; 5. 4x to 2; 6. 94 C 30”; 7. 25 C 3’; 8. +47 C at 0.25 C/sec; 9. 72 C 1’30”; 10. 94 C 30”; 11. 68 C 30”; 12. 72 C 1’30”; 13. 94 C 30”; 14. 68 C 30”; 15. 72 C 1’30”; 16. 94 C 30”; 17. 44 C 1’; 18. 72 C 1’30”; 19. 14x to 10; 20. 72 C 5’; 21. 4 C Hold; 22. End). Dilute a portion of the primary reaction 1:50 and use as template for secondary reaction with the DNA template replaced by the diluted primary reaction and TAIL1 replaced by the TAIL2 primer. The program is as follows (1. 94 C 20”; 60 C 30”; 3. 72 C 1’30”; 4. 94 C 20”; 5. 60 C 30”; 6. 72 C 1’30”; 7. 94 C 20”; 8. 44 C 1’; 9. 72 C 1’30”; 10. 11x to 1; 11. 72 C 5’; 12. 4 C Hold; 13. END). A tertiary PCR reaction is then performed with a 1:50 dilution replacing the DNA template and TAIL3 replacing TAIL2 with the following program (1. 94 C 20”; 2. 44 C 30”; 3. 72 C 1’30”; 4. 19x to 1; 5. 72 C 5”; 6. 4 C HOLD; 7. END). At this point, there will be a primary, secondary, and tertiary reaction for 3 random primers yielding 9 total reactions. 4 µL samples of those reactions are run on a 1% agarose/1x TAE gel for analysis. Ideally, a band that gets progressively shorter through each reaction will be visible, and sequencing of this band will yield the desired sequence.
2.3 Clone Generation

2.3.1 Sterilize Seeds

In generating hairy root lines, the use of sterile conditions is vital. It must be maintained throughout and requires the initial sterilization of seeds using bleach and Triton. Initially, place seeds in a 1.5 mL microfuge tube. Add 1 mL of 50% bleach/0.02% Triton X100. Shake for 5 minutes at room temperature. Spin briefly to gather seeds at bottom and remove liquid with pipetman. Add 1 ml sterile distilled water, vortex the tube. Repeat spin step, liquid removal, and washing with water. Again, remove as much water as possible. The seeds are now ready for immediate planting.

2.3.2 Planting and Infection

Sterile plant germination media is made by autoclaving. 1 L of solid germination media includes 20 g sucrose, 6 g agar, and 1 L Gamborg's B5 basal salt mixture (Sigma G5768). Before autoclaving, the pH should be adjusted to 5.7. Autoclave the mixture for 20 minutes and place in 55 °C water bath for cooling. Within sterile laminar flow hood, add 1 mL sterile Gamborg's vitamin solution (Sigma G1019) and mix. Immediately, add about 80 mL to previously sterilized magenta boxes by pipet and allow media to solidify. Once the media has solidified or at least reached a thick gel state, seeds can be sowed. Approximately nine seeds can be placed in each box and should be pushed approximately 1 cm down into the media. This is easily achieved by wetting a sterile wooden culture streaker with the solidified media, picking up the seeds on the end, and pushing them into the media. These boxes should then be placed in a dark 26 °C room for germination.
After approximately two weeks, the plants should be moved to a growth room with a 16 hour light/8 hour dark cycle. In an additional three weeks, the seedlings will be ready for infection. With the desired strain of *A. rhizogenes* grown in YEM media including antibiotics (i.e. kanamycin 50 mg/L) at 28 °C for approximately 36 hours, sterile surgical scissors are dipped in the 5 mL culture and used to cut the seedlings near the stem tip. Additionally, sterile razor blades can be dipped and used to further wound the seedlings and spread *A. rhizogenes*. The infected plants are then placed at 26 °C in the dark overnight before returning them to the 16 hour light/8 hour dark cycle. At three weeks, hairy roots should be visible. At five to six weeks post-infection, the hairy roots should be ready for excision.

### 2.3.3 Selection of Hairy Root Clones

Hairy roots are excised from the plants and immediately placed in Petri plates with solidified growth media. In order to assist in the removal of bacterial contamination, the roots should be pushed gently into the media. The media is prepared just as described for the solid germination media except the sucrose concentration is 30 g/L and salts are added at half strength. After cooling, the appropriate antibiotics in addition to the vitamins are added. The inducible glucocorticoid promoter reported here contains a hygromycin selection marker, and therefore hygromycin is added at a final concentration of 30 mg/L from a 15 mg/mL stock. Cefotaxime was also added at a concentration of 250 mg/L from a 125 mg/mL stock. At four weeks, lines showing substantial growth
were moved to plates lacking antibiotics. If further growth was noted and no contamination was present, lines from this step were used for liquid adaptation.

### 2.3.4 Liquid Adaptation

The adaptation of hairy root lines is a very sensitive process during which the majority of lines will die. It is important to consider this as one scales the original planting for clone generation. In addition to the 9 to 12 months of completely sterile work required to generate transgenic lines, this should be a vital concern. Including losses associated with germination, successful hairy root excision, growth on selection media, and long-term liquid adaptation rates, the final transformation efficiency was around 1%. The rates were reduced for some genes and higher for others but the number can be used as a guide to set reasonable expectations. The least successful step though is often the liquid adaptation of cultures which relies heavily on observation.

At this point, a number of lines have been selected that grow on selection media. Approximately four to five of the longest tips (longer than 4 cm) should be removed from the solid culture and placed in 50 mL liquid media. Ideally, these tips will be fast-growing, straight, white tips. The filter-sterilized liquid media includes 30 g/L sucrose, half strength Gamborg's B5 basal salts, and full strength Gamborg's vitamins with the pH adjusted to 5.7. The 250-ml Erlenmeyer flask containing these tips is then shaken at 100 rpm and 26 °C in the dark. Approximately four weeks is a suitable growth time, but some cultures will take longer and others less time. With the emergence of multiple long tips, the cultures should be recultured. Reculturing of these tips during their growth
stage and before much yellowing proves vital in maintaining the line. Even so, lines seem to show transients in growth during the first few generations and therefore the selection of appropriate tips is vital. After four to five generations, the behavior of each clone should be more predictable. At this point, cultures can be put on a standard culture cycle and used for experiments. Standard culture conditions include the use of a fixed number of tips of a relatively constant length subcultured at fixed time points. While a cycle of four weeks and five tips of 4 cm length can be used as a guide, successful liquid adaptation is dependent on the observation-dependent adaptation of those parameters.

2.4 Molecular Characterization of Clones

2.4.1 Northerns

Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen) as previously described. RNA was quantified by spectrophotometer according to absorbance at 260 and an ethidium bromide stained test gel used to verify the quality of RNA. 12 μg of total RNA were loaded per well and northerns performed essentially according to a reference protocol (Brown & Mackey 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen QIAEX II kit. Probes were then made using the Stratagene Prime-It II kit.
2.4.2 Genomic PCR

In certain cases noted within the text of this thesis, northern analysis was inconclusive. As a means of testing the insertion of desired genes, PCR was performed against genomic DNA preparations. These PCR results were then used as additional proof or preliminary evidence. The inducible promoter encodes two vital genes. The first is a glucocorticoid-activated transcription factor (GVG). The second is the gene of interest behind yeast GAL4 upstream activating sequences. To confirm the presence of GVG, PCR was performed with two primers spanning its coding sequence (GVG1: ATGAAGCTACTGTCTTCTATCGAACAAGCA. GVG2: GGCAGTCATTTTTGATGAAACAGAAGCTT). For insertion of the desired inducible gene, a primer specific to the GAL4 activating sequences (CONFGAL1 AGCTTGCATGCCGGTGACCTCTAGAGGATCC) and one specific to the 3’ end of the inserted gene were used.

2.4.3 Enzyme Assays

Anthranilate synthase enzyme assays were performed essentially as previously reported (Last & Fink 1988). After a 72 hour induction at 3 μM with uninduced controls exposed to equal amounts of ethanol, approximately 1 g of fresh weight four week old biomass was harvested and ground in a mortar and pestle with grinding buffer (200 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 8 mM MgCl2, 0.2 mM DTT, 60% glycerol), 200 mg glass beads, and 200 mg polyvinylpolypyrrolidone. All steps were performed at 4 °C unless otherwise noted. Supernatant was cleared by centrifugation and desalted on a Bio-Rad Econo-Pac 10DG column pre-equilibrated in column buffer (50 mM Tris-HCl, pH
7.5, 0.05 mM EDTA, 2 mM MgCl₂, 0.05 mM DTT, 5% glycerol). AS activity was measured in a 2.0 ml reaction with 1 ml column buffer plus 0.05 mM chorismic acid, 10 mM glutamine, 1 mM MgCl₂, and 12.5 mM tris-HCl (pH 8), and 20 μg crude protein as determined by Bradford Assay (Bradford 1976). Tryptophan from a 10 mM stock was added at the desired concentration to the assay volume to test the inhibition of the anthranilate synthase activity. The reaction was terminated after 30 minutes at 30 °C by adding 0.2 mL of 1.0 M HCl. The anthranilate produced was extracted into 3.5 mL ethyl acetate and quantified on a fluorescence spectrophotometer (excitation 340 nm, emission 400 nm). Standard curves were generated by adding known amounts of anthranilic acid to the reaction assay volume without protein and following the same extraction procedure.

Tryptophan decarboxylase activities were measured as previously described (Sangwan et al 1998). Again, all procedures were carried out at 4 °C unless otherwise noted. 1 g of four week old hairy root tissue was extracted by mortar and pestle in 2 mL buffer (0.1 M sodium phosphate buffer pH 7.5, 5 mM β-mercaptoethanol, 5 mM thiourea) with 100 mg polyvinylpolypyrrolidone and 100 mg glass beads. The homogenate was cleared by centrifugation at 10000g for 30 minutes, and the supernatant used as the enzyme source. Protein concentrations were determined by Bradford assay and enzyme assays were performed with 15 μg of protein for 30 minutes at 30 °C. The 1 ml (pH 8.5) assay mixture contained 0.1 M sodium phosphate buffer, 3.5 mM β-mercaptoethanol, 1 mM L-tryptophan, and 1 mM pyridoxal-5'-phosphate. Reactions were terminated with 2.0 mL 4 M NaOH and extracted with 3.5 mL ethyl acetate.
Tryptamine was measured in the extract with appropriate standard curve by measurement of fluorescence with excitation at 280 nm and emission at 350 nm.

2.5 Metabolic Analysis

2.5.1 Standards

One of the primary difficulties associated with evaluation of the alkaloid pathways is the limited availability of standards from commercial sources. Within this thesis, ajmalicine (Fluka), serpentine (Aldrich), and tabersonine (ICN biomedicals) are used. Previous work in our laboratory has isolated lochnericine and hörhammericine suitable for retention time analysis. Since tabersonine, lochnericine, and hörhammericine share the same α-methyleneindoline chromophore, tabersonine standard curves are used for the determination of lochnericine and hörhammericine yields. Once the desirable range of concentrations has been determined, standards are made through serial dilution.

2.5.2 Harvesting Cultures

Hairy root cultures were removed from the shake flask, blotted dry, and weighed for the determination of fresh weight. Total media volume was measured by pipet and 5 mL stored. Both the media and cultures were then immediately frozen at −80 °C. When alkaloid analysis was desired, the cultures were lyophilized and weighed again for dry weight determination.
2.5.3 HPLC Analysis of Metabolites

A crude MeOH extract of each harvested culture was prepared by extracting 50 mg of powdered freeze-dried biomass with 10 mL of MeOH for an hour in a chilled sonicating bath. Extracts were clarified at 1300g for 15 min. at 15 °C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then passed through a 0.22 μm Nylon filter (13 mm). 10 μL of this extract was injected onto a Phenomenex Luna 5μ C18(2) HPLC column (250x4.6 mm) under two different solvent systems. The Thermo Separations Products Spectrasystem HPLC system used included a P4000 pump unit, an AS3000 autosampler, and a UV2000 detector. For the detection of tryptophan and tryptamine, a protocol was adapted from the literature with UV detection at 218 nm (Tikhomiroff & Jolicoeur 2002). For twelve minutes, a flow rate of 1 ml/min was maintained of a 15:85 mixture of MeCN:100 mM phosphoric acid. The column was then washed with an 85:15 mixture and reequilibrated. Another solvent system was used for the detection of the indole alkaloids ajmalicine, serpentine, hörhammericine, lochnericine, and tabersonine. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described. For the first five minutes, the mobile phase consisted of a 20:80 mixture of 50% MeOH/50% MeCN : 5 mM (NH₄)₂HPO₄ pH 6 pumped at 1 mL/min. Over ten minutes, it was linearly ramped to a 64:36 mixture where it was maintained for fifteen minutes. Over the next five minutes, the flow rate was linearly
ramped to 1.4 mL/min. The ratio was then increased to 80:20 over five minutes where it was maintained for 15 minutes. The column was then reequilibrated to the starting 20:80 mixture for injection of the next sample.
Chapter 3

Characterization of an Inducible Promoter System in Transgenic

*Catharanthus roseus* Hairy Roots

This chapter essentially as presented here has been published in

Biotechnology Progress, 2002 (Hughes et al. 2002)

3.1 Abstract

Transgenic hairy root cultures of *Catharanthus roseus* were established with a glucocorticoid-inducible promoter controlling the expression of green fluorescent protein (GFP), and GFP expression was characterized. The inducible system shows a tightly controlled, reversible, and dosage-dependent response to the glucocorticoid dexamethasone in *C. roseus* hairy roots. Full induction was noted after twelve to eighteen hours in the mature regions of the root tips and after six hours in the meristem tissue. Upon removal of the inducing agent, GFP expression declined to undetectable levels in the mature tissues after 24 hours and in the meristem after 48 hours. Although no dosage-dependent response was noted in the meristem region, it was apparent in the mature region of the tip and was verified by quantitative GFP analysis. The inducible promoter system allowed quantitative control of GFP expression between 0.01 and 10μM dexamethasone with saturation occurring at higher levels. Using GFP as a model system, the ability to control temporal and quantitative gene expression with the glucocorticoid-inducible promoter in transgenic *C. roseus* hairy roots was demonstrated.
3.2 Introduction

*Catharanthus roseus* is a source of several valuable alkaloids including the chemotherapeutic agents vincristine and vinblastine. As traditional means of increasing metabolic flux through a pathway, including media optimization and elicitation, have been unable to overcome the tight regulation governing the production of these valuable pharmaceuticals in *C. roseus* (Hughes & Shanks 2002), researchers are now utilizing the techniques of metabolic engineering to address these limitations.

Commonly used experimental systems in *C. roseus* include hairy roots and cell cultures. Transgenic work to date has focused primarily on cell cultures using constitutive promoters. Hairy roots, which are caused by *Agrobacterium rhizogenes* infection, offer several advantages over cell cultures. Although both hairy roots and cell cultures exhibit fast growth, hairy roots are known to accumulate significantly higher levels of alkaloids (Moreno-Valenzuela et al 1998). Additionally, hairy roots have demonstrated improved genetic (Baiza et al 1999) and biochemical stability (Deus-Neumann & Zenk 1984; Shanks & Morgan 1999). Although numerous wild-type *C. roseus* hairy root lines have been studied, the establishment and successful maintenance of transgenic *C. roseus* hairy roots had been unsuccessful (Hallard et al 1997) until a recent report (Ayora-Talavera et al 2002). While all wild-type hairy roots have incorporated foreign T-DNA genes from the Ri plasmid of *A. rhizogenes*, lines described as transgenic within this article designate lines that also carry an additional gene of interest.
Previous work has firmly established the activity of the constitutive cauliflower mosaic virus (CaMV) 35S promoter in *C. roseus* (Ayora-Talavera et al 2002; Canel et al 1998; Goddijn et al 1995; van der Fits & Memelink 2000), but an inducible promoter could offer significant advantages. Clonal variation often presents a significant problem to data interpretation in transgenic studies. It can be difficult to separate the effects of clonal variation from the effects of specific gene over-expression. Negative controls in constitutive promoter studies are independently transformed lines, but an inducible promoter utilizes the same line as a negative control thereby minimizing the problems of line variation (Berlin & Fecker 2000). Furthermore, constitutive expression of genes often leads to deleterious effects whose impact can be difficult to separate from the direct effects of the over-expressed gene. Retarded growth is a deleterious effect often encountered in cell culture that selects against those lines with high transgene expression. By separating the growth and production phases, an inducible promoter may improve gene expression stability in cell culture (Verpoorte et al 2000). Inducible promoters also allow for the investigation of developmental effects. Previous work has demonstrated that alkaloid levels in hairy roots respond differently to precursor feedings depending on their growth phase (Morgan & Shanks 2000). An inducible promoter allows one to control the timing of the expression and therefore enables the study of these temporal effects. Gene expression levels can also be controlled by the dosage of the inducing agent. Overall, inducible promoters offer significant flexibility for a variety of metabolic studies.
While a variety of inducible promoters have been developed, their activities have not been demonstrated in *C. roseus*. The glucocorticoid-inducible promoter chosen for this study has previously shown high induction, a dosage dependent response, and low basal expression levels in tobacco (Aoyama & Chua 1997; Nara et al. 2000), *Arabidopsis* (Aoyama & Chua 1997), and rice (Ouwerkerk et al. 2001). The promoter system includes two components: the transcription factor GVG that is constitutively expressed behind the CaMV 35S promoter and the gene of interest cloned behind GAL4 upstream activation sequences. The GVG transcription factor is composed of a yeast GAL4 DNA-binding domain, the Herpes VP16 activation domain, and the rat glucocorticoid receptor (Aoyama & Chua 1997). In the absence of a ligand, the glucocorticoid receptor inactivates the transcriptional activator encoded by the DNA-binding and activation sequences. With the addition of dexamethasone, an artificial glucocorticoid hormone, the transcriptional activator is de-repressed and induces expression of the gene of interest. In this paper, we report the establishment of transgenic *C. roseus* hairy roots with dexamethasone-inducible expression of green fluorescent protein (GFP). Within the study, GFP provides a model system allowing for the characterization of the inducible promoter in our system including quantitative protein expression data.

### 3.3 Materials and Methods

#### 3.3.1 Establishment of Hairy Root Cultures. The plasmid pTA007 (Aoyama & Chua 1997), containing the glucocorticoid inducible GFP construct, a hygromycin plant selection marker, and a spectinomycin bacterial selection marker was electroporated into
A. rhizogenes 15834 which already has the Ri plasmid. Transformed colonies from YEM plates (1 g/L yeast extract, 0.2 g/L NaCl, 0.2 g/L MgSO₄·7H₂O, 4 mg/L FeCl₃, 0.66 g/L K₂HPO₄·3H₂O, 10 g/L mannitol, 15 g/L agar) including spectinomycin (100 mg/L) were analyzed for the presence of the plasmid pTA007 by restriction digest. A single colony was then used to start a 5 mL culture that was grown at 28 °C and 200 rpm for 36 hours in liquid YEM + spectinomycin (100 mg/L) media. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described (Bhadra et al 1993). After six weeks, hairy roots were excised from the plants and selected on solid and liquid media as described except 30 mg/L hygromycin was added for the first solid subculture.

3.3.2 Root Tip Induction and Microscopy. For tip induction, root tips approximately 2.5-3 cm in length were excised from liquid grown cultures. The tips were placed in six well plates with 4 mL filter sterilized liquid B5 media (half strength salts, full strength vitamins, 30 g/L sucrose, pH 5.7). The indicated amounts of a filter sterilized 5 mM dexamethasone solution dissolved in ethanol were used for induction. The plates were then put on an orbital shaker at 100 rpm and 26 °C. For initial examination of induction, only one tip was used. After 60 hours in the presence of dexamethasone, this tip was washed and placed in fresh media lacking dexamethasone. This procedure was repeated at 64 hours to ensure the complete removal of dexamethasone. For the dosage response experiments, five tips were used at each dosage level, and serial dilutions of a 5 mM stock were made so that an equal volume of dexamethasone/ethanol solution was added to each tip. A previously established line lacking the GFP gene LBE-6-1 (Bhadra et al
1993) was used as a negative control for varying dexamethasone concentrations. GFP expression was observed at 10X magnification using a Zeiss Axiovert 135 microscope outfitted with a fluorescence source and a standard FITC filter set. Pictures were taken of the root meristem region and more mature tissue using an attached Panasonic GP-KR222 digital camera interfaced with Scion Image 1.62c. Mature tissue pictures were taken approximately 1-1.5 cm back from the meristem region.

3.3.3 Quantitative Analysis of GFP Expression Levels. Approximately 1.5 g FW of four-week-old biomass from the sixth liquid-adapted generation of line EHIGFP-M was placed in 50 mL liquid media adjusted to the appropriate dexamethasone dosage. Line LBE-6-1 was used as a GFP minus control. All data are the mean of triplicate cultures. The 250 mL Erlenmeyer flasks containing the induced biomass were placed on an orbital shaker at 100 rpm in a 26 °C temperature controlled room. At the appropriate time, the mass was harvested, briefly dried by blotting, and protein extracts were prepared by a published method (Remans et al 1999). The biomass was ground using a mortar and pestle in 4 mL of buffer (10 mM Tris-EDTA, pH 8.0, 200 mg/L sodium azide) at 4 °C. The extract was then clarified by centrifugation at 13000 rpm for 10 min at 4 °C. After recentrifuging the supernatant, it was adjusted to 550 μg protein/mL using extraction buffer according to the results of Bradford Assays (Sigma) with BSA as the standard. The supernatant was frozen at –80 °C for approximately one week, and 2 mL were assayed on a Biorad VersaFluor Fluorometer (excitation 490 nm, emission 510 nm) as previously described (Remans et al 1999). Using purified eGFP (Clontech) which is similar to the
GFP variant used (Kost et al. 1998), a standard curve was drawn with eGFP in the protein extracts of LBE-6-1. The relationship was linear with a slope of 840 RFU/ (μg eGFP/mg soluble protein).

3.4 Results and Discussion

3.4.1 Establishment of a GFP Expressing Line.

As some inducible systems do not work in all species (Zuo & Chua 2000) and no inducible promoter had been reported for *C. roseus*, the activity of the glucocorticoid inducible promoter within the experimental system had to be tested. Additionally, successfully maintained transgenic *C. roseus* hairy roots had not yet been reported (Ayora-Talavera et al. 2002; Hallard et al. 1997). Initially, 130 *C. roseus* (cv. Little Bright Eye) plants were infected with *A. rhizogenes* carrying the inducible GFP plasmid. Ninety-six hairy roots were excised from the plants after six weeks, and 26 of these grew successfully on solid selection media. Overall, three lines adapted successfully to liquid B5 media and showed strong GFP expression upon induction. As noted previously (Hallard et al. 1997), the adaptation of *C. roseus* hairy roots to liquid media is problematic and results in a significant reduction of viable lines. Line EHIGFP-1b was the first to be identified and was used for initial testing of the inducible promoter. As EHIGFP-M grew substantially faster, later studies used this line.

For initial verification of transformation, one tip of line EHIGFP-1b was induced at 1.5 μM dexamethasone. The time course of the resulting induction in mature and meristem tissue is shown in Figure 3-1. Mature tissue as used here refers to the root
Figure 3-1. Time course of GFP expression in the meristem region (A) and the mature region (B) of EHIGFP-1b hairy root cultures induced with 1.5 μM dexamethasone. Negative hours indicate time after removal of dexamethasone.
tissue located 1-1.5 cm back from the meristem tissue of each root tip. Weak GFP expression is apparent at 3 hours and 9 hours for the meristem and mature tissue, respectively. At 6 hours, the meristem tissue appears to be fully induced whereas it takes 12 hours for the mature tissue to reach full induction. Expression levels were tested after up to 60 hours in the presence of dexamethasone and found to remain stable throughout this period. Expression levels are highest in the fast-growing meristem region. To test the reversibility of the induction, the root tip was washed after 60 hours in the presence of dexamethasone. GFP fluorescence in the meristem tissue disappeared 48 hours after removal of the dexamethasone, whereas the mature tissue stopped fluorescing after 24 hours. These times are partially dependent on the half-life of GFP in these regions and therefore the preexisting protein levels at the time of washing, but they are consistent with previously reported results on the reversibility of this inducible promoter. In transformed tobacco, luciferase RNA levels rapidly decrease between 24 and 48 hours after transfer to dexamethasone free media with no luciferase RNA detectable after 72 hours (Aoyama & Chua 1997). The results presented here clearly illustrate the activity and reversibility of the glucocorticoid system in *C. roseus* hairy roots. Furthermore, GFP expression appears to be highly inducible with low basal expression.

### 3.4.2 Dosage Dependence

A desired characteristic of an inducible system is a range of responses controlled by the dosage of the inducing agent. In order to study this phenomenon, tips from the third liquid-adapted generation of line EHIGFP-M were exposed to dexamethasone
dosages between 0.1 and 30 μM over 102 hours (n=5). At the meristem, induction was obvious at 6 hours for all dosages between 0.1 to 30 μM. As expected, no fluorescence was visible in the majority of tips of EHIGFP-M exposed to ethanol or LBE-6-1 (GFP minus) at 30 μM dexamethasone. Across dosage levels, no difference in induction at the root meristem was visible (Figure 3-2). However, the mature root tissue showed a dosage dependent response (Figure 3-3). Induction in the mature tissue took 18 hours and showed a gradual increase in GFP expression from 0.1 μM up to 10 μM. At 30 μM, the expression was not distinguishable from that at 10 μM, suggesting possible saturation of the response. In addition to a dosage-dependent response, the saturation observed here at dosages above 10 μM dexamethasone has been reported in studies on transgenic tobacco (Aoyama & Chua 1997) and rice (Ouwerkerk et al 2001). Although these results clearly show a dosage response in the mature tissue, root tips at the same dosage showed a significant amount of variability. The meristem regions of a few tips even showed fluorescence at time zero or zero dosage indicating nonspecific GFP expression in particular tips of this line. Other researchers have reported leaky expression in select lines of rice (Ouwerkerk et al 2001), but line EHIGFP-M showed significant leakage only in the meristem regions of a few tips. While the study of particular tips demonstrated dosage dependence, the most common experimental system is a shake flask grown culture in which both the less intense but dosage dependent induction of the mature region and the sensitive and quick induction in the meristem contribute to the overall induction effect.
Figure 3-2. Dosage response of meristem region of EHIGFP-M root tips exposed to concentrations of dexamethasone from 0 to 30 μM over 102 hours. GFP minus line does not carry the GFP gene.
Figure 3-3. Dosage-dependent response of mature region of EHIGFP-M root tips exposed to concentrations of dexamethasone from 0 to 30 μM over 102 hours. GFP minus line does not carry the GFP gene.
3.4.3 Quantitative Expression Data

To obtain accurate induction curves and quantify the response in a flask culture, protein extracts were prepared and assayed on a fluorometer. As seen in Figure 3-4, the time profile of GFP expression in EHIGFP-M was monitored for 100 hours at a dosage rate of 30 μM. Strong induction is reached at 24 hours but GFP expression increases until 48 hours after which it remains stable until 100 hours. As expected, exposure of a GFP minus line to dexamethasone had no effect on background fluorescence. As a flask culture is mainly composed of mature biomass in the central region with root tip meristems on the periphery, the quantitative results should be more dependent on the induction of mature region expression. While the experimental conditions are different, the long induction time of 24 hours is consistent with the 18 hour induction time for mature tissue noted in Figure 3-3.

As control of expression levels is also desirable, protein extracts from cultures induced with differing dexamethasone concentrations were prepared 100 hours after induction. The results are presented in Figure 3-5. As expected, the background fluorescence of the GFP minus negative control does not vary with dosage rate. Line EHIGFP-M, however, exhibits dexamethasone dosage dependent levels of fluorescence. Between 0 μM and 10 μM, the assay reports increasing GFP levels and therefore confirms the dosage dependent response noted in the pictures of the mature tissue. Even at dosage levels as low as 0.01 μM, the system is responsive and shows induction. Saturation occurs at dexamethasone dosages above 10 μM, as previously noted in Figure 3-3. The ability to use low dosage rates could prove important as other researchers have
Figure 3-4. Time course of GFP induction for shake flask cultures of EHIGFP-M exposed to 30 μM dexamethasone. GFP minus line does not carry the GFP gene. RFU are relative fluorescence units with buffer set as zero. Data shown are means +/- standard deviation of three replicate experiments.
**Figure 3-5.** GFP expression levels at 100 hours for EG1GFP-M shake flask cultures induced at varying dexamethasone dosages. GFP minus line does not carry the GFP gene. RFU are relative fluorescence units with buffer set as zero. Data shown are means +/- standard deviation of three replicate experiments.
reported growth defects in some transgenic lines of *Arabidopsis* (Kang et al. 1999) and rice (Ouwerkerk et al 2001) grown at high dexamethasone concentrations, such as 10 μM. However, during the course of these experiments, no phenotypic effects of dexamethasone dosage on the *C. roseus* hairy roots were noted. Although possible dexamethasone dependent effects may affect the suitability of this system for some studies, the quantitative GFP data confirm that the promoter system has the desirable characteristics of temporal and quantitative gene expression control in our system.

### 3.5 Conclusion

Both the proven activity of the inducer and the ability to generate transgenic hairy roots reported here provide additional options for the metabolic engineering of the *C. roseus* system. As previously reported in other systems, the inducible glucocorticoid promoter system had the desirable characteristics of low basal expression, high inducibility, and a dosage dependent response. Furthermore, the ability to generate transgenic hairy root lines using this system enables the study of a metabolically engineered tissue culture known to produce high levels of alkaloids and to remain genetically stable. After over eight months in antibiotic-free media, the lines reported here still show strong GFP induction.

### 3.6 Acknowledgements

The authors would like to thank Dr. Nam-Hai Chua at the Rockefeller University for providing the inducible promoter plasmid (pTA007) and Dr. Eugene W. Nester at the
University of Washington for providing the *A. rhizogenes* 15834 strain used in this study. This work was supported by funding from the National Science Foundation (Grant Numbers BES-0003730, BES-9906978). Erik H. Hughes was supported by a training grant from the National Institutes of Health (T32-GM08362).
Chapter 4

Glucocorticoid-Inducible Expression of a Feedback-Resistant Anthranilate Synthase in Catharanthus roseus Hairy Roots

4.1 Abstract

Transgenic hairy root cultures of Catharanthus roseus (periwinkle) were established with a glucocorticoid-inducible promoter controlling the expression of an Arabidopsis feedback-resistant anthranilate synthase alpha subunit. Based on previous precursor feeding studies indicating a developmental dependence of alkaloid accumulation on tryptophan pools, a study to determine the effects of elevated pools during the late exponential and early stationary growth phases was undertaken. Inducible expression of the anthranilate synthase alpha subunit was tested and confirmed by both northern analysis and enzyme assays. At the transcriptional level, induction after three days at 3 μM dexamethasone was dramatic with no transcription noted in the uninduced control. The indication of tight control was corroborated by enzyme assays in which the uninduced inhibition curve mirrored that of a negative control line. Furthermore, the enzyme assays demonstrated that the Arabidopsis alpha subunit was compatible with the native beta subunit and that glutamine-dependent anthranilate synthase activity was more resistant to tryptophan inhibition in the induced extract. The metabolic effects of anthranilate synthase alpha induction were also dramatic. Over a six day induction during the late exponential phase, tryptophan and tryptamine specific yields increased
from almost undetectable levels to 2.5 mg/g DW and from 25 µg/g DW to 267 µg/g DW, respectively. Smaller increases in these metabolites were noted over three day inductions and also during the early stationary phase. While the alteration of indole pathway regulation was clearly successful, consistent effects on the derived terpenoid indole alkaloids were not present. There was, however, a statistically significant 81% increase in lochnericine after the three-day exponential induction indicating the possibility of increased alkaloid flux counterbalanced by tight regulatory control over alkaloid levels. This study indicates the suitability of the glucocorticoid system within \textit{C. roseus} for well controlled metabolic engineering studies, presents a successful method to overcome the strict regulation governing indole precursor pools within our system, and examines the role of increased indole pathway flux for the synthesis of terpenoid indole alkaloids.

\section*{4.2 Introduction}

\textit{Catharanthus roseus} (periwinkle) has been extensively studied due to its production of two valuable alkaloids, vincristine and vinblastine, which are used in chemotherapy. Studies have included efforts at increasing alkaloid flux through elicitation (Moreno et al 1993; Rijhwani & Shanks 1998), precursor feeding (Moreno et al 1993; Morgan & Shanks 2000), and attempts at media optimization (Bhadra et al 1993). While these studies have elucidated some characteristics of the complex regulation governing indole alkaloid production, it still remains a complex network with a number of uncharacterized and uncloned enzymes (Hughes & Shanks 2002). The genes that have been cloned and characterized however present researchers with an
opportunity to metabolically engineer the system, characterize the outcome, and therefore further elucidate the network.

The broad class of terpenoid indole alkaloids in *C. roseus* is formed by the coupling of secologanin from the terpenoid pathway and tryptamine from the indole pathway. Precursor feeding studies have occasionally found that feeding the indole precursors, tryptamine and tryptophan, leads to increased accumulation of indole alkaloids (Kreuger & Carew 1978; Zenk et al 1977). Specifically, studies on *C. roseus* hairy roots show that tryptophan feeding during the late exponential growth phase results in significant increases in the accumulation of two alkaloids, tabersonine and serpentine (Morgan & Shanks 2000). In *C. roseus* cell lines engineered to overexpress strictosidine synthase, which catalyzes the coupling of the indole and terpenoid moieties, tryptophan feeding combined with terpenoid feeding was necessary to further increase alkaloid synthesis after an initial terpenoid deficiency was overcome (Whitmer et al 2002a). While a systems approach rather than single gene studies will likely be necessary for substantial increases in alkaloid flux, only the indole pathway and its regulation have been extensively characterized. Studies have been reported in a number of plant systems including rice (Tozawa et al 2001), *Arabidopsis* (Li & Last 1996), and *C. roseus* (Poulsen et al 1993). In the first committed step of tryptophan synthesis, anthranilate synthase (AS) catalyses the conversion of chorismate to anthranilate and is feedback inhibited by tryptophan (Figure 1) (Li & Last 1996). The AS enzyme is a tetramer composed of two α subunits (ASα) and two β (ASβ) subunits. It is the α subunit that
Figure 4-1. Regulation of the indole pathway in *C. roseus*. Arrows with minus signs indicate previously identified steps sensitive to feedback regulation. AS, anthranilate synthase. TDC, tryptophan decarboxylase.
catalyzes the aromatization of chorismate and binds tryptophan as a feedback inhibitor, while the β subunit donates an amino group from glutamine.

In addition to precursor feeding indications, the increased transcription of indole pathway genes in plants has previously been associated with the increased synthesis of defensive secondary metabolites (Li & Last 1996; van der Fits & Memelink 2000). In C. roseus cell cultures, transcription of ASα is coordinately regulated with other alkaloid biosynthetic genes by ORCA3, a jasmonate-responsive transcriptional regulator (van der Fits & Memelink 2000). Upon transfer to media used for the induction of alkaloid production, AS specific activity is also known to increase (Poulsen et al 1993). The coordinate regulation of the indole pathway and the alkaloid pathways points to the pathway’s potential significance in supplying precursors for increased alkaloid synthesis. With the positive indications from regulatory and feeding studies, we sought to engineer the indole pathway within our experimental system, C. roseus hairy roots. Recent reports of an inducible promoter useful for C. roseus (Hughes et al 2002) and the generation of transgenic C. roseus hairy roots (Ayora-Talavera et al 2002; Hughes et al 2002) provide new options for pathway manipulation. The indole pathway however presents some challenges due to its tight regulation. Increased tryptophan levels inhibit native AS activity and induce chorismate mutase thereby channeling flux into a competitive pathway (Verpoorte et al 1999). Fortunately, the regulatory network has been overcome in a few studies through constitutive expression of a feedback-resistant ASα subunit (Cho et al 2000; Tozawa et al 2001). An Arabidopsis ASα gene from a mutant resistant to
tryptophan analogues has been cloned (Li & Last 1996) and should allow researchers to effect changes in free tryptophan levels and potentially indole derived metabolites.

In this paper, we report the generation of *C. roseus* hairy roots transgenic for a feedback-resistant *Arabidopsis* ASα subunit. The gene is cloned behind a glucocorticoid-inducible promoter that has previously been used successfully within our system and should provide an improved negative control against clonal variation. As precursor effects have been shown to be developmentally dependent, we study the effects of induction during both the late exponential and early stationary phases. At each point, we report the effects of ASα induction on tryptophan, tryptamine, and specific alkaloid levels.

4.3 Results

4.3.1 Clone Generation and Tightly Controlled Transcriptional Inducibility

*Agrobacterium rhizogenes* strain ATCC 15834 carrying the Ri plasmid and p7002ASA was used to infect approximately 350 five-week-old *C. roseus* seedlings. The p7002ASA plasmid contains the coding region of the wild-type *Arabidopsis* ASα gene with a point mutation to confer feedback resistance (Li & Last 1996). In addition to the ASα gene under the control of the glucocorticoid promoter, the plasmid also contains a hygromycin selection marker and a constitutively expressed glucocorticoid inducible transcription factor (GVG). Of the 161 hairy roots excised, 39 showed strong growth on solid selection media containing 30 mg/L hygromycin. Of these, only six adapted to long term maintenance of ten generations on liquid media. Negative control lines (NC) were
similarly generated with ATCC 15834 carrying the Ri plasmid and p7002 which lacks the inducible AS\(\alpha\) gene but contains all other elements of the inducible system. 379 seedlings were infected yielding 156 hairy roots of which 43 grew on solid media and four adapted to long term liquid culture maintenance.

After a minimum of four liquid generations, four NC and four AS\(\alpha\) lines were subjected to northern analysis to check for AS\(\alpha\) inducibility. Each line was induced at 3 \(\mu\)M dexamethasone from a 5 mM dexamethasone solution in ethanol for 72 hours. Equal amounts of ethanol were added to the uninduced controls. Total RNA isolated from these eight lines from both induced and uninduced flasks was hybridized with labeled AS\(\alpha\) cDNA, TDC, and GVG as the probes. Ubiquitin 3 from Arabidopsis was used as a loading control probe. Of those lines tested, two NC lines and two AS\(\alpha\) lines exhibited readily detectable GVG transcription levels in both induced and uninduced states. The two AS\(\alpha\) lines also showed induction of AS\(\alpha\) levels in the induced state with no background expression visible in the uninduced state. Of the two lines in each case, the one with higher overall GVG transcript levels and induction of AS\(\alpha\) were chosen for further analysis. The northerns for these two lines, EHISASA-1 and EHINC-12-1, are shown in Figure 4-2. As added verification, both lines were also subjected to genomic PCR to confirm insertion of the genes. The insertion of GVG was confirmed in both, and insertion of AS\(\alpha\) was confirmed in EHISASA-1 by using a primer specific to the promoter’s GAL4 upstream activating sequences and one at the 3’ end of the AS\(\alpha\) gene.
**Figure 4-2.** Northern analysis of lines EHINC-12-1 and EHIASA-1. Lines were induced (I) at 3 μM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 μg RNA was loaded and blotting performed. Probes were made for *Arabidopsis* anthranilate synthase α (ASA), tryptophan decarboxylase (TDC), the glucocorticoid transcription factor (GVG), and ubiquitin 3 (UBQ3).
4.3.2 Inducible ASα Leads to Reduced Feedback Sensitivity

Initially, the overall glutamine-dependent activities of the lines were measured. Desalted protein extracts were prepared as detailed and the specific activities of the uninduced and induced states compared. Glutamine-dependent activity is dependent on the compatibility of the transgenic *Arabidopsis* ASα with the native ASβ and the availability of ASβ subunits. Activity was measured *in vitro* by measurement of anthranilate produced over 30 minutes by 20 μg of crude desalted protein. On induction, there was a 10% increase in AS activity from 4.18 to 4.61 pmol/mg/s in EHINGC-12-1, the negative control line. The AS line, EHIASA-1, showed a 36% increase from 2.75 to 3.75 pmol/mg/s. While a substantial increase was noted, it was not as dramatic as one might initially expect from the transcriptional induction. Glutamine-dependent activity requires both α and β subunits though and therefore activity is not simply a function of ASα levels.

In order to further test the induction of the feedback-resistant ASα, tryptophan inhibition curves for the induced versus uninduced protein extract were generated. In Figure 4-3, the inhibited activity levels from uninduced and induced extracts of the NC and ASα lines are plotted as a percentage of their initial *in vitro* activity versus tryptophan concentration. The induced AS extract clearly shows an increase in feedback resistance. Furthermore, the results are promising in that the uninduced AS extract closely mirrors the results from the NC line indicating that nonspecific expression
Figure 4-3. Tryptophan inhibition curves of protein extracts for ASα (EHIASA-1) and NC (EHINC-12-1) lines. Lines were induced (I) at 3 μM for 72 hours and protein extracts prepared. Ethanol was added to uninduced controls. Data plotted are the percent of initial in vitro activity at a range of tryptophan concentrations.
using this inducible system as previously reported (Hughes et al 2002; Ouwerkerk et al 2001) is likely limited.

4.3.3 Inducible ASα Yields Large Increases in Tryptophan and Tryptamine

The use of a feedback-resistant ASα should enable continued enzymatic activity in the presence of increased tryptophan levels within the system. After confirming the transcriptional inducibility and altered enzymatic activity on dexamethasone treatment, we evaluated the effect of induction on tryptophan levels. Since differential effects have been noted in precursor feeding studies based on growth stage, points were taken after a 3 and 6 day 3 μM induction during both the late exponential and early stationary growth phases. Growth curves had previously been generated based on fresh weight measurements of actively growing cultures on a fixed subculture cycle.

The HPLC results show a dramatic increase in tryptophan levels upon induction. From initial levels that are hardly detectable, the induced AS line accumulates 2.5 mg/g DW after a six day induction in the exponential phase. As seen in Figure 4-4, the longer induction times lead to higher accumulations of tryptophan, and inductions during the exponential phase are more effective than those during the stationary phase. Induction has no substantial impact on tryptophan levels in the NC line indicating the increase is a clear consequence of ASα induction. While such an increase is the intended direct effect of our manipulation, the underlying motivation in studying C. roseus using an inducible promoter was to examine the effects of tryptophan accumulation on downstream metabolites in a well-controlled system. The significant and sudden metabolic change
along with the absence of any effects on the NC line indicates that such an analysis is possible.

Within our system, tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC). Northern analysis (Figure 4-2) and activity assays within our laboratory are consistent with literature reports that indicate high native levels of TDC transcription (Goddijn et al 1995) and activity in *C. roseus* hairy roots (data not shown). *In vitro* assays have shown TDC activity levels in hairy roots to be at least an order of magnitude higher than those found in cell culture (Moreno-Valenzuela et al 1998). Even in cell culture though, native TDC activity levels were found to be sufficient for the utilization of tryptophan for increased alkaloid production (Whitmer et al 1998). Assays of tryptamine levels in our system closely mirrored the trends in tryptophan levels while showing a much smaller percentage increase. Effects of induction on the NC line were again insignificant. Data for both the NC and AS lines are displayed in Figure 4-5. Six day inductions of the AS line resulted in higher tryptamine levels than those measured over three day inductions. Increases during the exponential phase were higher than those measured during the stationary phase indicating again that the metabolic activity of the line might have decreased with growth and nutrient levels. At all timepoints, the increase was greater than 3 fold with a maximum increase of 10 fold up to 0.27 mg/g DW. Elevated tryptophan synthesis on induction clearly impacted tryptamine levels as might be expected from the high native TDC activity levels already shown in hairy roots.
Figure 4-4. Tryptophan levels at various induction lengths and growth stages for ASα (EHIASA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Tryptophan was determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
Figure 4-5. Tryptamine levels at various induction lengths and growth stages for ASα (EHISAA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Tryptamine was determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
4.3.4 Transient Increase in One Alkaloid

Having successfully increased tryptophan and tryptamine levels, the effect of increased indole flux on the biosynthesis of terpenoid indole alkaloids was studied. While analysis of pathways this far removed is difficult due to the existence of secondary effects, a limited number of fixed timepoints, and the limited characterization of alkaloids and their pathways, five alkaloids were measured at each timepoint to evaluate the metabolic effects on alkaloids. These included tabersonine, hörhammericine, and lochnericine from the Aspidosperma family of alkaloids and ajmalicine and serpentine from the Coryanthe family. Tabersonine data has been omitted due to very low production in one of the lines making comparative analysis impossible. As shown in Figure 4-6, induction of the AS line caused no significant change in the levels of hörhammericine, ajmalicine, or serpentine. In contrast, a significant difference \((p<0.05)\) between the induced and uninduced AS line at three days was noted in lochnericine specific yield with no change noted in the NC line (Figure 4-7). After a three day 3 \(\mu\)M induction, lochnericine levels in induced cultures were 2.03 mg/g DW versus 1.12 mg/g DW in the uninduced state. NC lines showed no significant effects on alkaloids during the exponential growth stage (Figure 4-8), but slight browning in the NC lines induced during the stationary phase might indicate nonspecific elicitation from dexamethasone treatment as previously noted in other reports (Kang et al 1999; Ouwerkerk et al 2001). Accompanying this browning was a statistically significant effect on ajmalicine levels during the stationary phase (Figure 4-8). The browning noted in the NC line was not visible in the AS line and the related metabolic effects seem
Figure 4-6. Levels of alkaloids in ASα line. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Alkaloids were determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
Figure 4-7. Lochnericine levels in ASα (EHIASA-1) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Lochnericine was determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
Figure 4-8. Levels of alkaloids in NC line. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Alkaloids were determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
limited but should not be ignored in interpreting our results.

4.4 Discussion

4.4.1 The Inducible Promoter as an Advantageous Tool for Pathway Analysis

As no regeneration protocol has been reported for C. roseus, cell and tissue culture represent the only available options for metabolic engineering of the plant. Most work to date has focused on cell culture, but hairy roots have certain advantages. Differentiation is known to play a role in alkaloid accumulation, hairy roots contain higher levels of alkaloids than cell cultures (Moreno-Valenzuela et al 1998), and hairy roots maintain genetic and biochemical stability whereas cell cultures often do not (Baiza et al 1999; Deus-Neumann & Zenk 1984). Furthermore, the inducible promoter used within this study provides an improved negative control to evaluate the effects of any metabolic alteration. Negative controls in most studies are often different cell lines transformed independently. Here the negative control is the same genetic line without the inducing agent. Given that there is wide variability in alkaloid production in our system (Bhadra et al 1993), such a negative control allows for improved data analysis within the system. Furthermore, induction of the ASα gene within this study has shown that the inducible promoter allows the researcher to time a substantial metabolic change. In this study, a large metabolic change was reported at three days and nonspecific expression appeared insignificant. There also seemed to be minimal effects on alkaloid biosynthesis by the inducing agent. While some were noted in the stationary phase accompanying weak browning, they are likely due to the specific NC line chosen for this study.
Previous work had shown the activity of the promoter within our system. This study demonstrates the suitability of that system for studying the alkaloid pathways in *C. roseus*.

### 4.4.2 Successful Engineering of Indole Pathway

Overexpression of an *Arabidopsis* ASα subunit in *C. roseus* hairy roots led to substantial increases in both tryptophan and tryptamine levels. The *in vitro* enzyme assay results from the inhibition studies indicate that the *Arabidopsis* ASα subunit is compatible with the native ASβ subunit. While levels were almost undetectable in uninduced control cultures, tryptophan increased to 2.5 mg/g DW after a six day induction at 3 μM in the late exponential phase. Similar increases in tryptophan levels without huge changes in overall activity levels have been noted in other reports (Tozawa et al 2001). Along with the increase in tryptophan, tryptamine simultaneously rose 10 fold. TDC activity within *C. roseus* has previously been reported to be extremely high in hairy root cultures (Goddijn et al 1995; Moreno-Valenzuela et al 1998), and it appears that basal levels are sufficient to increase tryptamine levels when supplied with an increased tryptophan pool. As often occurs with downstream products, the increases were diminished. Such a result is consistent with reports that tryptamine feedback inhibits TDC activity (Noe et al 1984). Accumulated tryptamine may also be utilized for increased flux to the alkaloids or other metabolites. Two substantially increased peaks were noted in the chromatogram at 329 nm, and they have yet to be identified (Figure 4-9). Combined with results from other systems, the expression of a resistant ASα appears
sufficient to increase tryptophan pools for potential conversion into secondary metabolites. Such an approach could also prove useful for manipulating the accumulation of other valuable secondary metabolites in cases where there are indications that tryptophan supply may be a limiting factor (Silvestrini et al. 2002).

Our engineering of the indole pathway is subject to a notable secondary effect from auxins that has been hypothesized to play a role in alkaloid synthesis. A possible effect of increased tryptophan pools could be the synthesis of increased levels of auxin known to be a strong plant regulator with effects on particular alkaloid genes. The auxin IAA is known to be synthesized from tryptophan in even untransformed roots (Muller et al. 1998), hairy roots show increased sensitivity to auxins (Shen et al. 1990), and agropine A. rhizogenus strains insert genes for the synthesis of IAA (Cardarelli et al. 1985). In previous work though, the effects of auxins on alkaloids have been contradictory depending on the exact C. roseus system studied and auxin dosage (Aerts et al. 1992; Goddijn et al. 1992; Morgan & Shanks 2000). In our system, tryptophan or auxin feeding to C. roseus hairy roots increased thickening and branching (Morgan & Shanks 2000). At low levels of feeding, however, the effects on both alkaloids and morphology were limited. In this study, no such growth effects were noted indicating that the secondary effect is likely minimal. There have also been literature reports in Lemna gibba indicating that increased pools of L-tryptophan do not lead to significant increases in IAA levels (Baldi et al. 1991). The presence of such secondary effects however is worthy of note given the number of potential metabolic fates of tryptophan.
Figure 4-9. Chromatogram of uninduced and induced methanol extracts at 329 nm. Extracts pictured here were prepared from cultures treated for six days during the late exponential growth phase. * denotes a new peak that is substantially increased on induction.
4.4.3 Transient Alkaloid Increase Suggests Tight Regulation of Alkaloid Levels

Previous results have most often indicated that the terpenoid pathway is initially limiting to alkaloid accumulation in *C. roseus* (Merillon et al. 1986; Moreno et al. 1993; Morgan & Shanks 2000). However, some feeding studies have shown increases in alkaloids on tryptophan or tryptamine feeding (Kreuger & Carew 1978; Zenk et al. 1977). In *C. roseus* hairy roots, feeding tryptophan during the late exponential phase resulted in significant increases in serpentine and tabersonine after three days (Morgan & Shanks 2000). In the early stationary phase, no significant effects were seen three days later effectively demonstrating a transient effect. Within the work presented here, lochnericine specific yield increased 81% after a three day induction in the late exponential phase. No such effects were seen at the six day exponential phase timepoint or either stationary phase timepoint. While more detailed studies would be necessary to confirm the transient nature of the peak, the result is consistent with previous results. A feeding study on *C. roseus* cell culture reported a similar transient in that tryptamine increased the rate of alkaloid accumulation when combined with loganin feeding but not the final accumulation value (Whitmer et al. 2002a). The lochnericine spike is also consistent with the report of substantial increases in only certain alkaloids. It appears, therefore, that the effects of increased indole precursor availability include a limited impact on alkaloid accumulation. There could be an increase in indole alkaloid flux demonstrated by the transiently improved specific yields that is counterbalanced over longer time periods by strict regulation of alkaloid accumulation through catabolic pathways. More detailed
studies to explore the existence of these transient effects could be pursued by utilizing the 
flexibility of the inducible promoter.

4.4.4 Further Characterization and Multigene Efforts

As strictosidine synthase activities have been reported to be high in hairy roots 
(Meijer et al 1993b), a likely explanation of the limited alkaloid changes is that the 
availability of both indole and terpenoid precursors controls the long-term accumulation 
of alkaloids. Recent feeding studies using transgenic C. roseus cell lines have indicated 
such coordination is necessary (Whitmer et al 2002a; Whitmer et al 2002b). In both 
strictosidine synthase and tryptophan decarboxylase overexpressers, indole feeding 
combined with terpenoid feeding increased alkaloid yields after an initial terpenoid 
deficiency was overcome. It will be interesting within our system to use the inducible 
ASα line in combination with terpenoid feeding to see if the necessity of coordinate 
regulation holds. It also points out the importance of further characterization and 
engineering efforts focused on the non-mevalonate terpenoid pathway. As this pathway 
was only recently elucidated, characterization of the regulation and cloning efforts have 
just begun (Lichtenthaler 1999). A few attempts at engineering the pathway in other 
systems have been attempted, but progress is limited to date (Estevez et al 2001; 
Mahmoud & Croteau 2001). Potential limitations downstream of strictosidine synthase 
may also hinder gains in the particular alkaloids measured as previously noted in cell 
culture (Whitmer et al 2002a). Further work and characterization of these pathways
should, however, lead to an improved understanding of alkaloid regulation and metabolic engineering efforts.

4.5 Materials and Methods

4.5.1 Plasmid Construction

A full-length wild-type *Arabidopsis thaliana* ASA1 cDNA was kindly provided by Dr. Gerald R. Fink at Whitehead Institute for Biomedical Research. Based on the previous report of G to A substitution in the sixth exon of the ASA1 gene from tryptophan feedback-resistant *A. thaliana* (Li & Last 1996), a site-directed mutagenesis mediated by PCR was performed using overlapping mutagenic primers (5'-ttgcaAacctttgaagtttat-3' and 5'-aagggtTtgcaaatgtgccg-3'). The final PCR product was cloned into a pBluescript KS vector and sequenced to confirm the dominant point mutation. The mutated ASA1 cDNA was then inserted into XhoI/SpeI site downstream of GAL4-UAS promoter in the binary pTA7002 vector (Aoyama & Chua 1997). This construct was named p7002ASA.

4.5.2 Clone Generation

Plasmids described above were electroporated into *A. rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L Yeast Extract, 0.2 g/L NaCl, 0.2 g/L MgSO₄•7H₂O, 4 mg/L FeCl₃, 0.66 g/L K₂HPO₄•3H₂O, 10 g/L mannitol, 15 g/L agar) including kinnamycin (50 mg/L) were analyzed for the presence of the respective plasmid by restriction digest. A single colony was then used to start a 5 mL culture
grown at 28 °C and 200 rpm for 36 hours in liquid YEM + kanamycin (50 mg/L) media. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described. After six weeks, hairy roots were excised from the plants and selected on solid and liquid media as described except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest.

4.5.3 Culture Conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose, half strength Gamborg’s B5 salts, and full strength vitamins (pH 5.7). Cultures were initiated by placing five tips into a 250 Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Growth curves and residual media volumes were determined on fixed subculture cycle cultures by measuring the culture fresh weight and media volume every three days over a culture cycle. Induction was performed by adding the appropriate volume of a 5 mM dexamethasone stock in ethanol. An equal amount of ethanol was added to uninduced controls.

4.5.4 RNA Isolation and Northern Analysis

Hairy root flask cultures that were approximately four weeks old were used for transcriptional induction studies. A 5 mM stock solution of dexamethasone was used to induce each cell line with ethanol added to the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. RNA was quantified by spectrophotometer according to
absorbance at 260 and an ethidium bromide stained test gel used to verify the quality of RNA. 12 μg of total RNA were loaded per well and northerns performed essentially according to a reference protocol (Brown & Mackey 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen (Valencia, CA) QIAEX II kit. Probes were then made using Stratagene (La Jolla, CA) Prime-It II kit.

4.5.5 Enzyme Assays

Anthranilate synthase enzyme assays were performed essentially as previously reported (Last & Fink 1988). After a 72 hour induction at 3 μM with uninduced controls exposed to equal amounts of ethanol, approximately 1 g of fresh weight four week old biomass was harvested and ground in a mortar and pestle with grinding buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM MgCl₂, 0.2 mM DTT, 60% glycerol), 200 mg glass beads, and 200 mg polyvinylpolypyrrolidone. All steps were performed at 4 °C unless otherwise noted. Supernatant was cleared by centrifugation and desalted on a Bio-Rad (Hercules, CA) Econo-Pac 10DG column pre-equilibrated in column buffer (50 mM Tris-HCl pH 7.5, 0.05 mM EDTA, 2 mM MgCl₂, 0.05 mM DTT, 5% glycerol). AS activity was measured in a 2.0 ml reaction with 1 ml column buffer plus 0.05 mM chorismic acid, 10 mM glutamine, 1 mM MgCl₂, and 12.5 mM Tris-HCl (pH 8), and 20 μg crude protein as determined by Bradford Assay. Tryptophan from a 10 mM stock was added at the desired concentration to the assay volume to test the inhibition of the anthranilate synthase activity. The reaction was terminated after 30 minutes at 30 °C by
adding 0.2 mL of 1.0 M HCl. The anthranilate produced was extracted into 3.5 mL ethyl acetate and quantified on a fluorescence spectrophotometer (excitation 340 nm, emission 400 nm). Standard curves were generated by adding known amounts of anthranilic acid to the reaction assay volume without protein and following the same extraction procedure. Uninhibited AS assays were performed in triplicate and average values are reported.

4.5.6 HPLC Analysis

Harvested cultures were immediately frozen at −80 °C. After lyophilization, a crude MeOH extract was prepared by extracting 50 mg of freeze-dried biomass with 10 mL of MeOH for an hour in a sonicating bath. Extracts were clarified at 1300g for 15 min. at 15 °C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then passed through a 0.22 μm Nylon filter (13 mm). 10 μL of this extract was injected onto a Phenomenex (Torrance, CA) Luna 5μ C18(2) HPLC column (250x4.6 mm) under two different solvent systems. The Thermo Separations Products (San Jose, CA) Spectrasystem HPLC system used included a P4000 pump unit, an AS3000 autosampler, and a UV2000 detector. For the detection of tryptophan and tryptamine, a protocol was adapted from the literature with UV detection at 218 nm (Tikhomiroff & Jolicoeur 2002). For twelve minutes, a flow rate of 1 ml/min was maintained of a 15:85 mixture of MeCN:100 mM Phosphoric Acid. The column was then washed with an 85:15 mixture and reequilibrated. Another solvent system was used for the detection of the indole
alkaloids ajmalicine, serpentine, hörhammericine, lochnericine, and tabersonine. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described. For the first five minutes, the mobile phase consisted of a 20:80 mixture of 50% MeOH/50% MeCN : 5 mM (NH₄)₂HPO₄ pH 6 pumped at 1 mL/min. Over ten minutes, it was linearly ramped to a 64:36 mixture where it was maintained for fifteen minutes. Over the next five minutes, the flow rate was linearly ramped to 1.4 mL/min. The ratio was then increased to 80:20 over five minutes where it was maintained for 15 minutes.

4.6 Acknowledgements

The authors would like to thank Dr. Nam-Hai Chua at the Rockefeller University for providing the inducible promoter plasmid (pTA7002), Dr. Eugene Nester at the University of Washington for providing the A. rhizogenes 15834 strain used, and Dr. Gerald Fink at Whitehead Institute for Biomedical Research for providing the Arabidopsis ASA1 clone. This work was supported by funding from the National Science Foundation (Grant Numbers BES-0003730, BES-9906978). Erik Hughes was supported by a training grant from the National Institutes of Health (T32-GM08362).
Chapter 5

Metabolic Engineering of the Indole Pathway in *Catharanthus roseus*

Hairy Roots Using Inducible Expression of a Feedback-Resistant Anthranilate Synthase and Tryptophan Decarboxylase

5.1 Abstract

Transgenic hairy roots of *Catharanthus roseus* were established with glucocorticoid inducible tryptophan decarboxylase (TDC) expression alone or in combination with inducible expression of a feedback-resistant anthranilate synthase alpha subunit (ASα) from *Arabidopsis*. Northern analysis confirmed transcription of the anthranilate synthase gene on induction in the double line, and *in vitro* enzyme assays confirmed increased resistance to feedback inhibition by tryptophan. Although induction of TDC transcription was difficult to detect due to significant background expression, enzyme assays showed an increase of 48 % and 87 % in the TDC and double lines, respectively. Due to limited tryptophan pools and potentially increased utilization of excess tryptamine, the TDC line showed no significant increase in tryptamine levels on induction. When combined with ASα expression in the double line however, tryptamine levels increased as much as 6 fold for a 3 day late exponential induction indicating the potential necessity of upstream manipulations for increased tryptamine pools within *C. roseus* hairy roots. Interestingly, the TDC line showed an increase in serpentine specific yields of as much as 129 % on induction, but no effects on measured alkaloids were
noted in the double line. Both clones however have very different basal alkaloid biosynthetic capacities which may help to explain the differences in tryptamine utilization for alkaloid production. Within this study, the successful engineering of the indole pathway in *C. roseus* hairy roots is reported. Furthermore, the inducible promoter allows the study to be well controlled and therefore to explore the role of the indole pathway in alkaloid biosynthesis.

5.2 Introduction

*Catharanthus roseus* has warranted significant study due to its production of two valuable alkaloids used in chemotherapy. These alkaloids, vincristine and vinblastine, are produced at extremely low levels within the plants and remain resistant to feasible chemical synthesis due to their complex structures (Hughes & Shanks 2002; Verpoorte et al 1999). As a potential means of producing valuable secondary metabolites, *C. roseus* has been widely used as an experimental system for plant metabolic engineering. Since it is not a classical model organism, characterization of the incredibly complex alkaloid pathways and subsequent cloning of related genes has been slow (Verpoorte & Memelink 2002). Fortunately, a number of genes have been cloned and studies on relevant upstream pathways in other plant systems are progressing. The available genes are currently sufficient for a number of metabolic engineering attempts aimed at increasing flux to the alkaloids (Hughes & Shanks 2002).

In *C. roseus*, no suitable regeneration protocol has been established leaving cell and tissue culture as the only feasible experimental systems for transgenic studies. While
work to date on genetically increasing alkaloid flux in *C. roseus* remains limited, a few notable metabolic engineering attempts have been made that further elucidate the network. Of terpenoid indole alkaloid relevant pathways, previous attempts have included constitutive overexpression of tryptophan decarboxylase (TDC) in cell culture and crown gall calluses (Canel et al 1998; Goddijn et al 1995), HMG-CoA reductase in hairy roots (Ayora-Talavera et al 2002), the transcriptional regulator ORCA3 in cell cultures (van der Fits & Memelink 2000), and strictosidine synthase in cell culture (Canel et al 1998). Gains in particular indole alkaloids were made in the ORCA3, STR, and TDC cell culture studies under certain precursor feeding conditions (van der Fits & Memelink 2000; Whitmer et al 2002a; Whitmer et al 2002b). Most studies, however, still indicate a requirement for greater pathway and regulatory characterization and intensive efforts beyond the single gene approach. In addition to an increasing knowledge base, the development of new tools for plant metabolic engineering is encouraging. For instance, we believe that the availability of inducible promoters for plant systems could greatly facilitate the analysis of transgenic experiments (Zuo & Chua 2000). This is particularly true in systems such as ours with high clonal variation evident in alkaloid production (Bhadra et al 1993). Recently, we reported the activity of one such inducible promoter in *C. roseus* hairy roots (Hughes et al 2002). With continued characterization and new tools enabling improved studies, metabolic engineering may be able to overcome the current native restrictions on alkaloid biosynthesis in *C. roseus* but still remains a young field prone to slow development (Hughes & Shanks 2002).
Terpenoid indole alkaloids are synthesized by the coupling of the terpenoid secologanin with the indole tryptamine, which is directly synthesized from tryptophan by TDC. In previous studies, increased transcription and activity of AS and TDC have been associated with the increased accumulation of indole alkaloids (Meijer et al 1993b; Poulsen et al 1993; van der Fits & Memelink 2000). This coordinate regulation points to the importance of the indole pathway in providing additional precursors for alkaloid accumulation in our system. In addition to these positive indications, the availability of tools to engineer the pathway and a well-developed understanding of indole pathway regulation led to our current metabolic engineering study. AS has been found to regulate flux to tryptophan with overexpression of a feedback-resistant alpha subunit (ASα) leading to accumulation of tryptophan (Chapter 4). Within this paper, we report the engineering of the indole pathway using an inducible promoter in C. roseus hairy roots to control expression of TDC individually and in combination with feedback-resistant ASα. The effects of induction on tryptamine and indole alkaloids are reported, and the role of increased indole flux for improved alkaloid yields discussed.

5.3 Materials and Methods

5.3.1 Plasmid Construction

A full-length wild-type Arabidopsis thaliana ASA1 cDNA was kindly provided by Dr. Gerald R. Fink at Whitehead Institute for Biomedical Research. Based on the previous report of G to A substitution in the sixth exon of ASA1 gene from tryptophan feedback-resistant A. thaliana (Li & Last 1996), a site-directed mutagenesis mediated by
PCR was performed using overlapping mutagenic primers (5'-ttgcaAacccattgaagtttat-3' and 5'-aaggtTtgcaaatgtgcgcg-3'). The final PCR product was cloned into a pBluescript KS vector and sequenced to confirm the dominant point mutation. The mutated ASA1 cDNA was then inserted into XhoI/SpeI site downstream of GAL4-UAS promoter in the binary pTA7002 vector (Aoyama & Chua 1997). This construct was named p7002ASA.

Based on the DNA sequence data of the C. roseus TDC gene that contains no intron (DeLuca et al 1989b), the TDC gene was amplified from genomic DNA by PCR, and the PCR fragment was cloned into a pBluescript KS vector. DNA sequence of the cloned TDC agreed with that of the published TDC gene (Genbank accession No. X67662). This TDC gene was then inserted into XhoI/SpeI site downstream of GAL4-UAS promoter in the binary pTA7002 vector. This construct was named p7002TDC. The same inducible TDC gene was put into the p7002ASA construct by first amplifying a 775-bp fragment carrying the GAL4-UAS promoter, XhoI/SpeI cloning site, and pea rbc-3A terminator from p7002 vector using flanking primers containing a Sse8387I site at their 5' ends. After amplification, the PCR product was cut with Sse8387I enzyme and then inserted into a single Sse8387I site of pUC18 vector. This construct was named pUCGALA, and the sequence of the cloned insert was verified. Next, a 1.5-kb TDC gene cloned in pBluescript vector was isolated by XhoI and SpeI enzyme digestion and inserted into the same restriction site of pUCGALA to produce pUCGALATDC construct. Finally, a 2.3-kb Sse8387I fragment from pUCGALATDC was inserted into a single Sse8387I site of p7002ASA that is located near the right border of T-DNA. This
construct was designated p7002ASATDC in which expression of both ASA1 and TDC cDNAs is under the control of glucocorticoid-inducible GAL4-UAS promoter.

5.3.2 Clone Generation

Plasmids described above were electroporated into *A. rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L Yeast Extract, 0.2 g/L NaCl, 0.2 g/L MgSO$_4$$\cdot$7H$_2$O, 4 mg/L FeCl$_3$, 0.66 g/L K$_2$HPO$_4$$\cdot$3H$_2$O, 10 g/L mannitol, 15 g/L agar) including karnamycin (50 mg/L) were analyzed for the presence of the respective plasmid by restriction digest. A single colony was then used to start a 5 mL culture grown at 28 °C and 200 rpm for 36 hours in liquid YEM + karnamycin (50 mg/L) media. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described. After six weeks, hairy roots were excised from the plants and selected on solid and liquid media as described except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest.

5.3.3 Culture Conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose, half strength Gamborg’s B5 salts, and full strength vitamins (pH 5.7). Cultures were initiated by placing five tips into a 250 Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Growth curves and residual media volumes were determined on fixed subculture cycle cultures by measuring the culture fresh weight and media volume every three days over a culture cycle. Induction was performed by adding
the appropriate volume of a 5 mM dexamethasone stock in ethanol. An equal amount of ethanol was added to uninduced controls.

5.3.4 RNA isolation and Northern Analysis

Hairy root flask cultures that were approximately four weeks old were used for transcriptional induction studies. A 5 mM stock solution of dexamethasone was used to induce each cell line at 3 µM with ethanol added to the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen) according to manufacturer’s instructions. RNA was quantified by spectrophotometer according to absorbance at 260 and an ethidium bromide stained test gel used to verify the quality of RNA. 12 µg of total RNA were loaded per well and northerns performed essentially according to a reference protocol (Brown & Mackey 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen QIAEX II kit. Probes were then made using Stratagene Prime-It II kit.

5.3.5 Enzyme Assays

Anthraniolate synthase enzyme assays were performed essentially as previously reported (Last & Fink 1988). After a 72 hour induction at 3 µM with uninduced controls exposed to equal amounts of ethanol, approximately 1 g of fresh weight four week old biomass was harvested and ground in a mortar and pestle with grinding buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM MgCl₂, 0.2 mM DTT, 60% glycerol), 200 mg
glass beads, and 200 mg polyvinylpolypyrrolidone. All steps were performed at 4 °C unless otherwise noted. Supernatant was cleared by centrifugation and desalted on a Bio-Rad Econo-Pac 10DG column pre-equilibrated in column buffer (50 mM Tris-HCl, pH 7.5, 0.05 mM EDTA, 2 mM MgCl₂, 0.05 mM DTT, 5% glycerol). AS activity was measured in a 2.0 ml reaction with 1 ml column buffer plus 0.05 mM chorismic acid, 10 mM glutamine, 1 mM MgCl₂, and 12.5 mM tris-HCl (pH 8), and 20 μg crude protein as determined by Bradford Assay. Tryptophan from a 10 mM stock was added at the desired concentration to the assay volume to test the inhibition of the anthranilate synthase activity. The reaction was terminated after 30 minutes at 30 °C by adding 0.2 mL of 1.0 M HCl. The anthranilate produced was extracted into 3.5 mL ethyl acetate and quantified on a fluorescence spectrophotometer (excitation 340 nm, emission 400 nm). Standard curves were generated by adding known amounts of anthranilic acid to the reaction assay volume without protein and following the same extraction procedure.

Tryptophan decarboxylase activities were measured as previously described (Sangwan et al 1998). Again, all procedures were carried out at 4 °C unless otherwise noted. 1 g of four week old hairy root tissue was extracted by mortar and pestle in 2 mL buffer (0.1 M sodium phosphate buffer pH 7.5, 5 mM β-mercaptoethanol, 5 mM thiourea) with 100 mg polyvinylpolypyrrolidone and 100 mg glass beads. The homogenate was cleared by centrifugation at 10000g for 30 minutes, and the supernatant used as the enzyme source. Protein concentrations were determined by Bradford assay and enzyme assays were performed with 15 μg of protein for 30 minutes at 30 °C. The 1 ml (pH 8.5) assay mixture contained 0.1 M sodium phosphate buffer, 3.5 mM β-
mercaptoethanol, 1 mM L-tryptophan, and 1 mM pyridoxal-5'-phosphate. Reactions were terminated with 2.0 mL 4 M NaOH and extracted with 3.5 mL ethyl acetate. Tryptamine was measured in the extract with appropriate standard curve by measurement of fluorescence with excitation at 280 nm and emission at 350 nm. Both TDC activity assays and uninhibited AS assays were performed in triplicate and average values are reported.

5.3.6 HPLC Analysis

Harvested cultures were immediately frozen at –80 °C. After lyophilization, a crude MeOH extract was prepared by extracting 50 mg of freeze-dried biomass with 10 mL of MeOH for an hour in a sonicating bath. Extracts were clarified at 1300g for 15 min. at 15 °C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then passed through a 0.22 μm Nylon filter (13 mm). 10 μL of this extract was injected onto a Phenomenex Luna 5μ C18(2) HPLC column (250x4.6 mm) under two different solvent systems. The Thermo Separations Products Spectrasystem HPLC system used included a P4000 pump unit, an AS3000 autosampler, and a UV2000 detector. For the detection of tryptophan and tryptamine, a protocol was adapted from the literature with UV detection at 218 nm (Tikhomiroff & Jolicoeur 2002). For twelve minutes, a flow rate of 1 ml/min was maintained of a 15:85 mixture of MeCN:100 mM Phosphoric Acid. The column was then washed with an 85:15 mixture and reequilibrated. Another solvent system was used for the detection of the indole alkaloids ajmalicine, serpentine, hörhammericine,
lochnericine, and tabersonine. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericicine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described. For the first five minutes, the mobile phase consisted of a 20:80 mixture of 50% MeOH/50% MeCN : 5 mM (NH₄)₂HPO₄ pH 6 pumped at 1 mL/min. Over ten minutes, it was linearly ramped to a 64:36 mixture where it was maintained for fifteen minutes. Over the next five minutes, the flow rate was linearly ramped to 1.4 mL/min. The ratio was then increased to 80:20 over five minutes where it was maintained for 15 minutes.

5.4 Results and Discussion

5.4.1 Generation and Genetic Analysis of Lines

*Agrobacterium rhizogenes* strain ATCC 15834 carrying the Ri plasmid and p7002TDC was used to infect approximately 350 five-week-old *C. roseus* seedlings. The p7002TDC plasmid contains the coding region of the wild-type *C. roseus* TDC gene under the control of yeast GAL4 upstream activating sequences (UAS). It also contains a hygromycin selection marker and a constitutively expressed glucocorticoid inducible transcription factor (GVG) that binds the UAS and activates transcription in the presence of glucocorticoids. Of the 126 hairy roots excised, 22 showed strong growth on solid selection media containing 30 mg/L hygromycin. Of these, only two adapted to long term maintenance of more than ten generations on liquid media. 350 seedlings were also infected by ATCC 15834 carrying p7002ASATDC which contains an *Arabidopsis*
feedback-resistant anthranilate synthase alpha subunit reported earlier in addition to the TDC gene (Li & Last 1996). The TDC gene was cloned behind the GAL4 UAS of the inducible promoter and inserted into p7002ASA thereby making both ASα and TDC expression inducible. Of the 160 hairy roots excised, only 26 grew significantly on solid selection media. As previously reported (Bhadra et al 1993; Hallard et al 1997), the most difficult step is the adaptation of *C. roseus* hairy roots to liquid media and only two double lines successfully adapted to long term maintenance on liquid media. Negative control lines (NC) were similarly generated with ATCC 15834 carrying the Ri plasmid and p7002 which lacks any inducible gene but contains all other elements of the inducible system.

After a minimum of four liquid adapted generations, four negative control lines, two TDC lines, and two double ASα+TDC lines were subjected to northern analysis to check for transcriptional inducibility. Each line was induced at 3 μM dexamethasone from a 5 mM dexamethasone stock solution in ethanol for 72 hours. Equal amounts of ethanol were added to the uninduced controls. Total RNA isolated from these eight lines from both induced and uninduced flasks was hybridized with labeled ASα cDNA, TDC, and GVG as the probes. Ubiquitin 3 from *Arabidopsis* was used as a loading control probe. Two NC lines and all four TDC and ASA+TDC lines showed visible GVG transcription levels in both induced and uninduced states. The northerns from the TDC line EHITDC-15-2, the ASα+TDC line EHIASTDC-35-1, and the NC line EHINC-12-1 which were chosen for further study are shown in Figure 5-1. As evidenced by the NC results and uninduced results, TDC transcript levels in *C. roseus* hairy roots are very
high. This result is consistent with previous literature reports (Goddijn et al 1995). While ASα induction is clear in the double line, the background transcription of TDC makes the inducibility of this gene hard to detect. As an additional check, the insertion of the feedback-resistant ASα and TDC genes were confirmed by genomic PCR with primers specific to the GAL4 UAS and the 3' end of the appropriate gene.

5.4.2 Increased Enzymatic Activity on Induction

The TDC clone, EHITDC-15-2, and double clone, EHIASTDC-35-1, which grew the fastest, were selected for enzymatic characterization. Previous work with a single ASα clone showed that the most noticable change after induction was a change in the inhibition pattern of the AS activity rather than in total activity. In these studies, induction was again performed for 72 hours at 3 μM and enzyme extracts were prepared. Glutamine dependent AS activity increased 10% in the NC line on induction from 4.18 to 4.61 pmol/mg/s and 58% in EHIASTDC-35-1 on induction from 4.18 to 6.60 pmol/mg/s. In order to verify the resistance of AS to tryptophan feedback inhibition, inhibition curves were generated for the NC and AS lines. The extract from the induced double line showed a substantial increase of resistance over the uninduced extract and both extracts of the NC lines. This increased resistance can be seen in Figure 5-2 where AS activity is plotted as a percentage of initial activity at a range of tryptophan concentrations.
Figure 5-1. Northern analysis of lines EHINC-12-1, EHITDC-15-2, and EHIASTDC-35-1. Lines were induced (I) at 3 μM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 μg RNA was loaded and blotting performed. Probes were made for *Arabidopsis* anthranilate synthase α (ASA), tryptophan decarboxylase (TDC), the glucocorticoid transcription factor (GVG), and Ubiquitin 3 (UBQ3).
Figure 5-2. Tryptophan inhibition curves of protein extracts for double line (EHIASTDC-35-1) and NC (EHINC-12-1) line. Lines were induced (I) at 3 μM for 72 hours and protein extracts prepared. Ethanol was added to uninduced controls. Data plotted are the percent of initial in vitro activity at a range of tryptophan concentrations.
Crude protein extracts were assayed for TDC activity according to a recently published protocol (Sangwan et al. 1998). As previously reported and consistent with the high transcription level, TDC activity levels were very high in *in vitro* assays. On induction with dexamethasone, TDC activity levels in the NC line changed very little from 151 to 148 pmol/mg/s. In EHitDC-15-2, activity increased 48% from 125 to 186 pmol/mg/s. In the double line, the increase was 87% from 118 to 220. While basal levels of activity are certainly high, a substantial increase in TDC activity levels was noted in both lines transgenic for inducible TDC.

### 5.4.3 Increase in Tryptamine in ASα Line

Growth curves were generated based on fresh weight from hairy roots on fixed time subculture cycles. From these curves, the exponential and stationary phase growth phases were determined. During the late exponential phase and early stationary phase, individual flask cultures were induced at 3 μM dexamethasone for three or six days with ethanol added to uninduced negative controls. With only TDC expression, there were few effects on tryptamine concentration. For the 6 day exponential induction and both stationary phase inductions, there was no difference in tryptamine levels between induced and uninduced controls (Figure 5-3). At the three day exponential induction, there was an increase from 26.5 to 49.4 μg/g DW but it was not statistically significant (p<0.05). This dramatically contrasts the results reported for *C. roseus* crown gall calluses and cell cultures which accumulated tryptamine (Canel et al. 1998; Goddijn et al. 1995). It is not necessarily unexpected though as basal TDC levels are significantly higher in hairy roots...
than in either of those systems (Goddijn et al 1995; Moreno-Valenzuela et al 1998). Tryptophan levels were also below detection limits indicating that pools of precursors may be severely limited and that only limited tryptophan is available for the increase in TDC activity. Tryptophan has previously been reported as undetectable in other studies on *C. roseus* hairy roots (Tikhomiroff & Jolicoeur 2002). As indicated in Figure 5-3, no effects were noted in the NC lines on tryptamine pools. Furthermore, no significant effects on growth were noted in any line.

The absence of increased tryptamine pools on TDC induction may indicate that an improved availability of tryptophan is necessary for increased tryptamine levels. The double line, EHIASTDC-35-1, contains both an inducible feedback-resistant ASα and inducible TDC. At each timepoint, tryptamine pools were increased above the uninduced controls. During the exponential phase, tryptamine pools increased from 13.3 to 79.4 μg/g DW at three days and from 13.3 to 40.7 μg/g DW at six days. Increases were also noted in the stationary phase. In both the NC and double lines, tryptophan levels were below detection limits. The lack of change in tryptophan pools is surprising as inducible ASα clones in our laboratory have shown dramatic increases in tryptophan levels as well as tryptamine levels. This difference could be due to the biochemical activity of the particular clone, the availability of indole precursors in the line, a greater pull effect from increased TDC activity, or simply the fact that transcriptional induction was not nearly as dramatic in this line. Nevertheless, our results indicate that one must alter the indole pathway upstream to effect changes in tryptamine levels. While divergent from results obtained in other *C. roseus* systems, the difference can be explained by the high
Figure 5-3. Tryptamine levels at various induction lengths and growth stages for NC (EHINC-12-1), TDC (EHITDC-15-2), and ASα+TDC (EHIASTDC-35-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced (I) at 3 μM and harvested after 3 or 6 days. Ethanol was added to uninduced controls (U). Tryptamine was determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
native level of TDC activity of *C. roseus* hairy roots. Alternatively, increased utilization of tryptamine flux could account for the lack of tryptamine accumulation.

### 5.4.4 Inducible Increase in Indole Alkaloids

Synthesis of indole alkaloids is dependent on the availability of both the indole precursor tryptamine and the terpenoid precursor secologanin. While many studies indicate the terpenoid pathway is generally limiting (Whitmer et al 1998), results have been contradictory. Certain studies have shown increased alkaloid levels upon feeding of indole precursors (Kreuger & Carew 1978; Zenk et al 1977). In our system, tabersonine and serpentine increased upon tryptophan feeding during the late exponential growth phase (Morgan & Shanks 2000). The motivation in using the inducible promoter was that it provides a good negative control to study the effects of upstream modifications on alkaloid accumulation. Constitutive expression studies often present problems due to high clonal variation in indole alkaloid production and the variability of expression levels.

Five alkaloids were studied upon induction at the same timepoints previously analyzed for tryptamine content. No significant changes in the levels of tabersonine, lochnericine, hörhammericine, or ajmalicine were noted at any timepoint for the TDC clone as shown in Figure 5-4. However, significant (*p*<0.05) increases were noted in serpentine specific yields at all four time points with no similar changes in the NC clone (Figure 5-5). After the three day exponential induction, serpentine levels increased from 411 μg/g DW to 817 μg/g DW. As shown in Figure 5-5, changes at the six day time
point were less significant than the three day induction for both the exponential and stationary phase inductions. Two previous studies have linked increased specific yields of serpentine with the feeding of tryptophan (Morgan & Shanks 2000; Zenk et al 1977). Previous reports in cell cultures have also noted an effect of tryptamine on the alkaloid accumulation rate but not the final accumulation (Whitmer et al 2002a). The lessened effect at 6 days could be due to a transient effect of induction which diminishes over longer induction times. Similar transient spikes in alkaloid specific yields seen in previous hairy root studies may indicate that an increase in indole flux results in immediate improvements in alkaloid yields that are eventually counterbalanced by the tight regulation of alkaloid accumulation including catabolic pathways. While no accumulation of tryptamine was noted in our study, an increase in flux through tryptamine due to increased TDC activity is the most likely explanation for the gains in serpentine.

In the double line, no increases in measured alkaloids were noted as shown in Figure 5-6. While tryptamine increased in this line, it doesn’t appear that any tryptamine was used for the increased synthesis of measured indole alkaloids. The vast difference in alkaloid profiles between the two lines is worthy of note. The double line produces over 1 mg/g DW of serpentine and hörhammericine and about 2 mg/g DW of ajmalicine. By contrast, the uninduced TDC line produces only lochnericine at levels above 600 μg/g DW. It is clonal differences such as these that often make constitutive studies hard to
**Figure 5-4.** Levels of alkaloids in TDC line. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced (I) at 3 μM and harvested after 3 or 6 days. Ethanol was added to uninduced controls (U). Alkaloids were determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
Figure 5-5. Serpentine levels in TDC (EHITDC-15-2) and NC (EHINC-12-1) lines. Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced at 3 μM and harvested after 3 or 6 days. Serpentine was determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
Figure 5-6. Levels of alkaloids in double line (EHIASTDC-35-1). Late exponential (Exp) and early stationary (Stat) growth phase cultures were induced (I) at 3 μM and harvested after 3 or 6 days. Ethanol was added to uninduced controls (U). Alkaloids were determined by HPLC analysis from crude extracts. Data represents mean of triplicate cultures +/- standard deviation.
interpret and demonstrate the power of the inducible promoter. While it is difficult to
determine the exact flux changes using only a snapshot of five metabolites, differences in
the biosynthetic capacities of the two lines may help explain the divergent results. While
increased TDC activity levels led to improved specific yields of serpentine, increased
TDC activity levels combined with ASα levels showed no improvement. As noted in
other studies, perhaps it is the presence of a positive epigenetic background that is most
vital (Whitmer et al 1998). The role of indole pathway regulation is also unclear in these
results. ASα is normally feedback inhibited by tryptophan and weakly by tryptamine,
and TDC activity has been reported to be feedback inhibited by elevated tryptamine
levels. To further hypothesize about the effects observed in our study, more
characterization would prove necessary. However, we have shown that in a well-
controlled study that the flux to tryptamine within C. roseus hairy roots can be increased.
Furthermore, manipulation led to significant increases in the yields of one alkaloid.

Achieving large increases in alkaloid yields will likely require multigene studies
and significant further characterization of the pathways of interest. Of particular interest
is the nonmevalonate pathway for terpenoid synthesis (Lichtenthaler 1999). Feeding
results including those in our laboratory have implicated this pathway as limiting the
availability of precursors for alkaloid synthesis (Moreno et al 1993; Morgan & Shanks
2000). Furthermore, the necessity of complementing the indole precursor with the
terpenoid precursor in a controlled manner has proven vital to increased alkaloid
synthesis (Whitmer et al 2002a; Whitmer et al 2002b). Therefore, the current
characterization and metabolic engineering attempts targeted at the nonmevalonate
pathway are vital to further gains (Estevez et al 2001; Mahmoud & Croteau 2001). By combining the approach used in this study with methods affecting terpenoid synthesis, more substantial gains can likely be made. The method presented here might also prove important in other systems where the indole precursor may be limiting to the production of valuable secondary metabolites (Silvestrini et al 2002).

5.5 Summary

This paper reports the successful engineering of the indole pathway of *C. roseus* hairy roots using an inducible promoter. The method presented increased serpentine in one line and could likely further increase alkaloid synthesis when combined with other manipulations in a multigene study. Furthermore, the inducible system used presents an advantageous system to analyze the role of the indole pathway in alkaloid synthesis.

5.6 Acknowledgements

The authors would like to thank Dr. Nam-Hai Chua at the Rockefeller University for providing the inducible promoter plasmid (pTA7002), Dr. Eugene Nester at the University of Washington for providing the *A. rhizogenes* 15834 strain used, and Dr. Gerald Fink at Whitehead Institute for Biomedical Research for providing the *Arabidopsis* ASA1 clone. This work was supported by funding from the National Science Foundation (Grant Numbers BES-0003730, BES-9906978). Erik Hughes was supported by a training grant from the National Institutes of Health (T32-GM08362).
Chapter 6

A Precursor and Inhibitor Study on the Role of the Non-Mevalonate Terpenoid Pathway in Alkaloid Synthesis

The data presented in this chapter will be published in Biotechnology Progress.

6.1 Abstract

Terpenoid feeding in a number of Catharanthus roseus studies has led to increases in indole alkaloid accumulation indicating that terpenoid supplies may be limiting. The discovery of the non-mevalonate or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway and data suggesting that it is the primary source of isopentyl diphosphate (IPP) for alkaloid synthesis make the pathway an attractive one for further characterization and potential metabolic engineering. While numerous studies have explored alkaloid accumulation in the presence of additional terpenoid precursors, this study looks farther up the pathway to explore the role of the DXP pathway. Treatment with the antibiotic fosmidomycin, a specific inhibitor of DXP reductoisomerase, significantly reduced accumulation of three measured alkaloids in C. roseus hairy root cultures during the exponential growth phase. Conversely, feeding with the upstream terpenoid precursor 1-deoxy-D-xylulose and the downstream precursors 10-hydroxygeraniol or loganin resulted in significant and similar increases in alkaloid production for exponential phase hairy root cultures. These results suggest that the DXP pathway plays a crucial role in regulating carbon flow for the biosynthesis of monoterpenoids used to produce indole alkaloids in C. roseus hairy roots.
6.2 Introduction

*Catharanthus roseus* has warranted significant study due to its production of pharmaceutically valuable compounds used in the treatment of cancer, hypertension, and circulatory disorders (Leveque et al. 1996; Verpoorte & Memelink 2002). Unfortunately, the secondary metabolites of interest are produced at very low levels. As classical methods including media optimization, elicitation, and feeding studies have produced limited gains, researchers have begun to use molecular methods to improve the production system (Hughes & Shanks 2002). Significant effort has therefore been focused on increasing our understanding of the biochemical pathways by which the terpenoid indole alkaloids (TIAs) are produced and exploring the regulation that restricts their accumulation. Such an improved understanding should provide valuable guidance for the metabolic engineering of *C. roseus* for increased production of the TIAs.

TIAs are all derived from a shared intermediate that is formed by the coupling of the monoterpenoid secologanin with the indole tryptamine. Two pathways are therefore responsible for supplying precursors for increased alkaloid synthesis within our system. Most feeding studies however indicate that at least initially the supply of precursors from the terpenoid pathway limits alkaloid accumulation (Morgan & Shanks 2000; Whitmer et al. 2002a; Whitmer et al. 2002b). In plants, monoterpenes are derived from isopentyl pyrophosphate (IPP), the five-carbon precursor of all isoprenoids, which is synthesized either via the cytosolic mevalonate pathway or the plastidic 1-deoxy-D-xylulose 5-phosphate (DXP) pathway (Figure 6-1) (Lichtenthaler 1999). As most monoterpenes
Figure 6-1. Overview of the biosynthetic pathways leading to the terpenoid indole alkaloids of *C. roseus*. Underlined compounds were fed to hairy root cultures in this study and compounds in bold were quantified.
appear to be formed in the plastids, the DXP pathway should be expected to supply IPP for TIA biosynthesis as has been confirmed in NMR studies of *C. roseus* and *R. serpentina* (Contin et al 1998; Eichinger et al 1999).

The condensation of pyruvate and glyceraldehyde-3-phosphate to produce DXP is catalyzed by DXP synthase (DXS) and represents the first step in the DXP pathway. DXP is then converted into 2-C-methyl-D-erythiol-4-phosphate (MEP) by DXP reductoisomerase (DXR) which is subsequently used to produce IPP through a series of enzymatic steps. Within *C. roseus*, some promising data has been accumulated regarding the regulation of the two genes under conditions leading to increased alkaloid synthesis. While no visible transcription is noted for *C. roseus* cell suspension cultures in maintenance media, both DXS and DXR are strongly transcribed in alkaloid production media (Chahed et al 2000; Veau et al 2000). DXS expression is also induced by the jasmonate responsive transcriptional regulator ORCA3 which is known to increase alkaloid synthesis in cell culture (van der Fits & Memelink 2000). While a number of alkaloid bisynthetic genes are similarly regulated by both media transitions and ORCA3, the corresponding induction of the genes with an increase in alkaloid synthesis points to their possible role in supplying increased levels of terpenoid precursors. Transgenic studies have not yet been reported in *C. roseus* with either DXS or DXR but overexpression in other plants increases levels of some terpenoids. DXS overexpression enhances various terpenoids in *Arabidopsis* (Estevez et al 2001), and DXR overexpression increases oil yields in peppermint (Mahmoud & Croteau 2001). Previous
work in *E. coli* has also isolated these steps as important regulatory points in the synthesis of carotenoids (Kim & Keasling 2001; Matthews & Wurtzel 2000).

Feeding of mevalonic acid to *C. roseus* hairy roots in a previous study did not affect the accumulation of TIAs indicating that the increased availability of precursors from the mevalonate IPP pathway is probably not limiting (Morgan & Shanks 2000). This study focuses on the importance of the DXP pathway for alkaloid accumulation and the potential that metabolic engineering upstream could affect alkaloid yields. With this in mind, we examined the effects of the DXR enzyme inhibitor fosmidomycin, 1-deoxy-D-xylulose, and monoterpenoid precursors on the production of TIAs in *C. roseus* hairy roots. Here we report that during the exponential growth phase fosmidomycin significantly inhibited TIA synthesis and 1-deoxy-D-xylulose significantly increased TIA accumulation.

### 6.3 Materials and Methods

#### 6.3.1 Chemicals and treatments

Fosmidomycin (Molecular Probes, Eugene, OR) was dissolved in 10 mM Tris-HCl (pH 8.5) and added to cultures at a final concentration of 100 μM. 1-Deoxy-D-xylulose (Omicron Biochemicals, South Bend, IN) and 10-hydroxygeraniol (Aldrich, Milwaukee, WI) were added directly as liquids to a final concentration of 104 and 52 μM, respectively. Loganin (Apin Chemicals, Oxon, UK) was dissolved in water and added to a final concentration of 52 μM. Fosmidomycin and terpenoid precursors were added at 17 days after culture inoculation, and cultures were harvested at 21 days for late
exponential stage feedings. Dosages were doubled and feeding was performed at 21 days with harvesting at 25 days for stationary phase timepoints.

6.3.2 Culture conditions

Growth of the hairy root line LBE-6-1 used for this experiment was essentially as previously described (Morgan & Shanks 2000). Every 21 days, five tips were inoculated into 50 ml filter-sterilized medium (3% sucrose, half-strength Gamborg’s B5 salts, and full strength Gamborg’s vitamins, pH 5.7) in a 250-ml Erlenmeyer flask. The cultures were shaken at 100 rpm and kept at 26 °C in the dark. The fresh and dry weights of the cultures were determined after blotting and lyophilization, respectively.

6.3.3 Alkaloid analysis

The cultures were harvested and processed essentially as described before. Lyophilized roots were pulverized, and 50 mg of tissue were extracted twice with 10 ml methanol in a sonicating bath at 16 °C for 1 h. The extracts were clarified by centrifugation, combined, evaporated at 50 °C under vacuum to a 2-ml volume, and filtered through 0.2 μm nylon membranes. Ten μls of the filtered extract were then injected into the HPLC system to quantify alkaloids as previously reported (Morgan et al 2000).
6.4 Results and Discussion

6.4.1 The effects of precursor feeding

In order to improve our understanding of the DXP and terpenoid pathways, a precursor feeding with the three compounds noted by underlining in Figure 6-1 was performed. In this study, the concentration of deoxyxylulose, 10-hydroxygeraniol, and loganin used did not have any noticeable effects on growth. Unphosphorylated 1-deoxy-D-xylulose was used in this study because previous reports indicate that it is efficiently utilized for the biosynthesis of phytol and carotenoids in *C. roseus* cell cultures and rescues the albino phenotype of an *Arabidopsis* DXS mutant (Arigoni et al 1997; Estevez et al 2000). In our work, deoxyxylulose feeding of *C. roseus* hairy roots increased accumulation of tabersonine and lochnericine 30% and 21% above the control, respectively (Figure 6-2). Furthermore, similar increases in tabersonine and lochnericine were observed from equal 10-hydroxygeraniol and loganin feedings (Table 6-1). As previously reported (Morgan & Shanks 2000), 10-hydroxygeraniol and loganin feeding did not significantly affect ajmalicine accumulation.

6.4.2 The effects of fosmidomycin

The availability of the specific DXR inhibitor fosmidomycin provided another means of examining the role of the DXP pathway in providing monoterpenoids for alkaloid synthesis. Previous NMR work had demonstrated that the non-mevalonate DXP pathway was the primary method of secologanin and loganin synthesis in *C. roseus* and *R. serpentina* cell suspension cultures, respectively (Contin et al 1998; Eichinger et al
1999). If the DXP pathway is responsible for providing the majority of the terpenoid precursors, inhibition of DXR should significantly reduce indole alkaloid production. We therefore dosed hairy roots with the antibiotic fosmidomycin at 100 μM, a concentration that is known to specifically and completely inhibit DXR enzyme activity in higher plants (Fellermeier et al 1999; Rodriguez-Concepcion et al 2001; Zeidler et al 1998). No significant differences in morphology or fresh weight to dry weight upon treatment were noted. Consistent with the DXP pathway’s role as the primary provider of terpenoid precursors, fosmidomycin significantly inhibited the accumulation of ajmalicine, tabersonine, and lochnerericine during the exponential phase (Figure 6-3). During this phase, the accumulation of each alkaloid was inhibited by 31% to 57% as compared to the untreated control (Table 6-1). When the percentage inhibition is calculated using the new alkaloid production from day 17 to day 21 assuming no turnover of preexisting alkaloids from day 17, the inhibition is as high as 93% for ajmalicine. While the results during the exponential phase are convincing, alkaloid accumulation during the stationary growth phase was not significantly inhibited which may indicate the reduced metabolic activity of the tissue.

6.4.3 Analysis

Both deoxyxylulose feeding data and fosmidomycin inhibitor data point to the importance of the DXP pathway for the biosynthesis of TIAs in C. roseus hairy roots. The fosmidomycin data is consistent with previous NMR data indicating that the DXP
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<th>Alkaloids</th>
<th>Inhibitor</th>
<th>Terpenoid Precursor</th>
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<tr>
<td></td>
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</tr>
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</tr>
<tr>
<td>Lochnericine</td>
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<tr>
<td>Ajmalicine</td>
<td>-57*</td>
<td>31</td>
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Table 6-1. Percent changes in alkaloid specific yield with precursor feeding or inhibition as compared with control cultures. Triplicate cultures were fed at day 17 and harvested at day 21 (late exponential phase) with untreated controls harvested at day 21. * indicates p<0.05.
Figure 6-2. The effects of precursor feeding on the levels of alkaloids during exponential growth phase. NC, control; DX, deoxypyxylulose; 10HG, 10-hydroxygeraniol; LOG, loganin. Each bar represents mean with error represented as +/- standard deviation.
Figure 6-3. The effects of fosmidomycin inhibition on the levels of alkaloids during exponential and stationary growth phases. Gray bars indicate control cultures and black bars indicate inhibited cultures. Each bar represents the mean with error represented as +/- standard deviation.
pathway is the primary source of terpenoids for alkaloid biosynthesis. The positive impact of deoxyxylulose feeding points to DXS’s possible role in regulating terpenoid production within the hairy roots and indicates that metabolic engineering of the DXP pathway could benefit overall indole alkaloid productivity.

6.5 Conclusion

As previously reported in other systems, the DXP pathway plays an important role in the synthesis of terpenoids which are utilized in *C. roseus* for indole alkaloid production. Results reported here demonstrate that feeding with an early DXP pathway product increases indole alkaloid levels. Therefore, increasing the activities of the DXS/DXR enzymes may provide an effective strategy for the metabolic engineering of alkaloid production in *C. roseus*. 
Chapter 7

Preliminary Studies on the Engineering of Alkaloid Yields Through Manipulation of a Transcriptional Activator and Terpenoid Pathway Genes

7.1 Introduction

*Catharanthus roseus* has been extensively studied due to its production of pharmaceutically valuable indole alkaloids. Among other factors that control the limited production of these secondary metabolites, many studies have pointed to the importance of the terpenoid pathway. The monoterpenoid indole alkaloids are synthesized by the condensation of the indole tryptamine and the monoterpenoid secologanin, but precursor feeding studies have indicated that limitations in terpenoid precursors initially prohibit higher alkaloid yields (Whitmer et al 1998; Whitmer et al 2002a; Whitmer et al 2002b). While the data presented in this chapter is preliminary, it is valuable to consider as an early attempt in the manipulation of terpenoid metabolism and its effects. In addition to reporting preliminary efforts at manipulating terpenoid metabolism through single gene efforts, the effects of a transcriptional activator known to induce a variety of alkaloid biosynthetic genes will be explored (van der Fits & Memelink 2000).

Until recently, it was believed that the mevalonate pathway provided the terpenoid precursors for alkaloid synthesis. The elucidation of a new non-mevalonate, however, led to studies indicating that it rather than the mevalonate pathway provided the IPP used for alkaloid synthesis (Contin et al 1998; Lichtenthaler 1999). The first step of
the pathway is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) which couples pyruvate and glyceraldehyde-3-phosphate. While the regulation of this new pathway is still largely unexplored, genetic manipulations have led to improved production of specific carotenoids in *E. coli* and terpenoids in *Arabidopsis* (Estevez et al 2001; Kim & Keasling 2001; Matthews & Wurtzel 2000). Feeding the non-phosphorylated product of DXS has also led to increased carotenoid production in tomato (Lois et al 2000) and indole alkaloid production in *C. roseus* hairy roots (Chapter 6). The coordinate regulation of DXS with improved alkaloid content also points to the gene’s potential importance in regulating flux. For instance, cell cultures grown in alkaloid production media show much higher alkaloid yields and DXS transcription levels than those in maintenance media (Chahed et al 2000). While the regulation still requires elucidation, the DXS gene has been cloned allowing for metabolic engineering attempts.

Another recently cloned enzyme that has received overwhelming attention is geraniol 10-hydroxylase (G10H). Within the last few years, two new reports detail the cloning of the gene and its induction in cell suspension cultures on the transfer to alkaloid production media (Collu et al 2002; Collu et al 2001). Older reports also hypothesize its importance in cell suspension cultures. Two papers demonstrate a relationship between G10H activity and the ability of the cells to synthesize indole alkaloids (Dagnino et al 1995; Schiel et al 1987). Another study noted that G10H activity and indole alkaloid accumulation increased after treatment with phenobarbital whereas both decreased with ketoconazole treatment (Contin et al 1999). A US patent (5735507) was even filed
reporting the cloning of a G10H from *Arabidopsis* and pointing to its potential use in engineering plants for improved indole alkaloid production. It is important here to note that hairy roots are a different system that may have altered metabolic limitations. In fact, a previous study indicated that native plant roots contained the highest levels of G10H activity (Meijer et al 1993b). This observation is particularly relevant as hairy roots often share metabolic activities similar to native roots. Furthermore, a previous feeding study in *C. roseus* hairy roots found no significant difference in the alkaloid improvement from 10-hydroxygeraniol and geraniol (Morgan & Shanks 2000). This feeding study therefore pointed to upstream limitations in that the substrate and product of G10H were equally used. The goal of the work presented here was to present a preliminary attempt at testing the hypothesized importance of G10H.

Given the difficulty of generating significant gains from single gene studies, certain researchers have pointed to transcription factors as a way to modulate the expression of many genes simultaneously (Memelink et al 2001). Previous elicitation studies have successfully increased *C. roseus* alkaloid accumulation (Moreno et al 1993; Rijhwani & Shanks 1998), and jasmonic acid has been shown to transcriptionally activate a number of genes. Recently, a jasmonate responsive transcription factor was cloned (van der Fits & Memelink 2000), and constitutive expression in cell culture resulted in induced transcription levels of numerous alkaloid related genes. A 3-fold increase in indole alkaloids over controls was also noted but only after terpenoid feeding. While a number of genes were induced, other key genes including G10H were not induced and the cells were deficient in their production of terpenoid precursors. While such factors
may provide a valuable tool to simultaneously induce a number of genes, their limitations must be recognized. They may not induce all necessary genes, could activate catabolic pathways, and might elicit responses that are detrimental to growth.

The recent cloning of DXS, G10H, and ORCA3 genes enables further metabolic engineering studies and particularly efforts at manipulating terpenoid metabolism. The following short report discusses the generation and preliminary metabolic characterization of *C. roseus* hairy root lines transgenic for these three genes.

### 7.2 Results and Discussion

#### 7.2.1 DXS

Due to initial difficulty in obtaining a cDNA clone of *C. roseus* DXS, a fully characterized clone from *Arabidopsis* was used (Estevez et al 2001; Estevez et al 2000). The gene was put into the glucocorticoid inducible plasmid, transferred to *Agrobacterium rhizogenes* 15834, and used for the generation of transgenic hairy root lines. A particularly fast growing clone, EHIDXS-4-1, showing strong induction of DXS transcription was chosen for further analysis. Northern analysis for the constitutive transcription factor (GVG), inducible DXS, and Ubiquitin 3 as the loading control is presented in Figure 7-1 for both uninduced and induced cultures. As was the standard procedure, cultures including the negative control line EHIINC-12-1 were induced at 3 μM dexamethasone with ethanol added to the uninduced clones. RNA samples were prepared from fresh tissue at 72 hours and 12 μg loaded for northern analysis. As induction of the negative control line has no effects on the genes discussed here or the
accumulation of alkaloids during the exponential phase, discussion of those results has been omitted. The relevant data on those topics is presented in Chapters 3 and 4 of this thesis.

Metabolic analysis of EHIDXS-4-1 was performed in triplicate with induction at 3 μM during the exponential growth phase. Cultures were harvested at 3 days due to significant browning of the culture and media on dexamethasone induction possibly due to nonspecific elicitation. Harvested cultures were analyzed by HPLC for alkaloid content and the data is presented in Table 7-1. Ajmalicine and serpentine increased over 25%, but tabersonine, lochnericine, and hörhammericine all decreased dramatically. The chromophore of the latter three is visible at 329 nm and the chromatogram of uninduced and induced methanol extracts are pictured in Figure 7-2. There is obviously a dramatic new peak in the chromatogram that shows a similar UV spectra to tabersonine with the λmax is shifted to 336 from 329 nm under HPLC conditions. A new peak was also noted in the LCMS analysis of crude extracts. A comparative PDA analysis of that peak with a tabersonine standard is shown in Figure 7-3, and the LCMS analysis is displayed in Figure 7-4. These new peaks might represent a conversion product of tabersonine and could therefore be responsible for the diminished yield of tabersonine related alkaloids. While the large peak noted in the chromatogram at 329 nm could also be a product symptomatic of the severe browning, no dramatic new peaks were noted in a few negative control flasks that had previously shown severe browning during a stationary phase induction. In order to test the dependence of the results on the browning, a lower dosage study was undertaken at 0.5 μM dexamethasone. Cultures were harvested after
six days with very limited browning. Even with the dramatically reduced browning, the harvested cultures showed the same alkaloid effects and the dramatic new chromatogram peak at 329 nm. It therefore seems unlikely that the changes are simply an effect of nonspecific browning. If one quantifies the new peak using the tabersonine standard assuming the presence of a similar chromophore, the specific yield of this compound increases from undetectable levels to 1.9 mg/g DW on induction. Overall, the alkaloid yield across the six compounds individually quantified would then remain constant with uninduced cultures and induced cultures accumulating 4.5 and 4.6 mg/g DW, respectively.

Overall, the effects of DXS induction are unclear at this point. Significant increases in ajmalicine and serpentine are noted but other alkaloids decrease dramatically. New unidentified peaks are also observed in two types of analysis. The results differ dramatically from those observed with deoxyxylulose feeding in Chapter 6 where ajmalicine, lochnericine, and tabersonine all increased with no significant new peaks. One explanation for this difference could be that feeding increases the overall supply while DXS overexpression may put significant stress on central metabolism as it pulls on pyruvate pools. A low dexamethasone dosage study below the levels reported here could be used to study the effects of lower DXS induction levels and further reduce the browning reported in the study. Other methods useful in evaluating this clone could include pyruvate feeding and the simultaneous induction and fosmidomycin inhibition of the pathway. The development of methods to quantify terpenoid precursors is also vital as effects could be more dramatic in intermediate products given the metabolic distance
Figure 7-1. Northern analysis of lines EHINC-12-1 and EHIDXS-4-1. Lines were induced (I) at 3 μM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 μg RNA was loaded and blotting performed. Probes were made for *Arabidopsis* deoxyxylulose-5-phosphate synthase (DXS), the glucocorticoid transcription factor (GVG), and Ubiquitin 3 (UBQ3).
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<th>Alkaloid</th>
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<th>STD</th>
<th>Induced</th>
<th>STD</th>
<th>% Change</th>
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<tr>
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<td>32</td>
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</tr>
<tr>
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<td>199</td>
<td>353</td>
<td>44</td>
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</tr>
<tr>
<td>Hörhammericine</td>
<td>688</td>
<td>77</td>
<td>377</td>
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<tr>
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<td>1181</td>
<td>109</td>
<td>1520</td>
<td>51</td>
<td>27%*</td>
</tr>
<tr>
<td>Serpentine</td>
<td>348</td>
<td>46</td>
<td>434</td>
<td>33</td>
<td>25%*</td>
</tr>
</tbody>
</table>

**Table 7-1** Alkaloid analysis of DXS line. Cultures were induced for three days at 3 µM dexamethasone with ethanol added to the uninduced lines. Experiment was done in triplicate. * indicates p<0.05.
Figure 7-2. Chromatogram of DXS clone at 329 nm in uninduced and induced states. Crude methanol extracts were prepared from cultures treated for three days. Lochnericine (1), Tabersonine (2), Significant New Peak (3).
Figure 7-3. PDA spectra of Tabersonine (A) and unknown peak (B) visible on induction during LCMS analysis. Tabersonine elutes at 20 min., and new peak elutes at 40 min.
Figure 7-4. LCMS analysis of unknown peak visible in DXS line. Unknown peak under LCMS conditions elutes at 40 minutes.
between DXS and the indole alkaloids. The search for other increased peaks and their identification might also prove valuable in understanding the effects of DXS overexpression. As the effects of the induction were largely unexpected, there remain a number of important questions regarding this line.

7.2.2 G10H

During the process of liquid media adaptation, all prospective lines of G10H except for one marginally growing line were lost. At the time of writing, that clone, EHIG10H-17-3, appears to be growing much better than initially enabling further study. Northerns for G10H inducibility were performed exactly as previously reported for the DXS clones and are presented in Figure 7-5. Unfortunately, the induction of G10H isn’t obvious from the northerns. GVG transcription is apparent though. In order to test the integration of the genes, a PCR reaction was performed against a genomic DNA sample from EHIG10H-17-3 using a primer specific to the GAL4 upstream activating sequences used in the promoter system and a primer specific to the 3’ end of G10H. GVG integration was simultaneously tested and the integration of both was confirmed. Given the previous report that roots showed the highest levels of G10H, it is also relevant that basal G10H transcription appeared to be present in the northerns. Lanes for both negative control samples and the uninduced G10H clone possessed hybridization signals.

With the original weak growth of the line, sufficient biomass for triplicate experiments was not available. Alkaloid analysis on one induced and one uninduced culture during the exponential growth phase was performed. Cultures were induced at 3
Figure 7-5. Northern analysis of lines EHINC-12-1 and EHIG10H-17-3. Lines were induced (I) at 3 µM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 µg RNA was loaded and blotting performed. Probes were made for geraniol 10-hydroxylase (G10H), the glucocorticoid transcription factor (GVG), and Ubiquitin 3 (UBQ3).
<table>
<thead>
<tr>
<th>Alkaloid</th>
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<th>Induced Specific Yield (μg/g DW)</th>
<th>% Change</th>
</tr>
</thead>
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<tr>
<td>Tabersonine</td>
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</tr>
<tr>
<td>Lochnericine</td>
<td>922</td>
<td>896</td>
<td>-3%</td>
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<td>-4%</td>
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<tr>
<td>Ajmalicine</td>
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<td>901</td>
<td>18%</td>
</tr>
<tr>
<td>Serpentine</td>
<td>598</td>
<td>633</td>
<td>6%</td>
</tr>
</tbody>
</table>

**Table 7-2.** Alkaloid analysis of G10H line. Cultures were induced at 3 μM with ethanol added to the uninduced control. Crude methanol extracts were prepared from cultures treated for six days.
µM, harvested after six days, and alkaloids analyzed. The data from that analysis is presented in Table 7-2. The experiment should be repeated in triplicate to determine if the 64% change in tabersonine specific yield is significant. It will also be important to determine the inducibility of G10H transcription and activity through a new northern and in vitro enzyme activity assays. Alternatively, new lines that show stronger induction could be generated exactly as reported here. Even recognizing these qualifications, the increase in tabersonine could indicate the importance of increased G10H activity. It also mirrors the feeding results attained earlier in our system with 10-hydroxygeraniol feeding during the stationary growth phase. In that study, a 50% improvement in tabersonine yield was reported on 10-hydroxygeraniol feeding with little effect on the other measured alkaloids (Morgan & Shanks 2000). Further characterization including a triplicate study is necessary for further comment on this line and the potential importance of G10H.

7.2.3 ORCA3

A line with strongly inducible ORCA3 transcription was tested through northern analysis. EH1ORCA3-4-1, the line chose for further study, showed visible transcription of GVG and strongly inducible transcription of ORCA3. Those results are shown in Figure 7-6. With the integration and inducibility of the gene tested, a preliminary metabolite analysis was performed in triplicate. Cultures were induced at 3 µM dexamethasone and harvested after six days. Ethanol was added to the uninduced controls. HPLC analysis was then used to determine the effects of induction on alkaloid
Figure 7-6. Northern analysis of lines EHINC-12-1 and EHIORCA3-4-1. Lines were induced (I) at 3 μM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 μg RNA was loaded and blotting performed. Probes were made for ORCA3, the glucocorticoid transcription factor (GVG), and Ubiquitin 3 (UBQ3).
<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Uninduced Specific Yield (μg/g DW)</th>
<th>STD</th>
<th>Induced Specific Yield (μg/g DW)</th>
<th>STD</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
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<td>134</td>
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<td>10</td>
<td>-86%*</td>
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<tr>
<td>Hörhammericine</td>
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<td>752</td>
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<td>23%</td>
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<tr>
<td>Ajmalicine</td>
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<td>1055</td>
<td>123</td>
<td>998</td>
<td>85</td>
<td>-5%</td>
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</table>

**Table 7-3.** Alkaloid analysis of ORCA3 line. Cultures in triplicate were harvested after treatment for six days. Induction was performed with dexamethasone at 3 μM with ethanol added to uninduced cultures. * indicates p<0.05
accumulation in the line (Table 7-3). The only statistically significant change (p<0.05) is the 86% reduction in tabersonine specific yields. While this report may seem contradictory to the positive expectations, it may simply be a reflection of some problems with elicitation and the use of transcription factors regulating the response.

Although they used different elicitation substances, two studies in cell suspension culture reported no increase in indole alkaloid accumulation (Moreno et al 1996; Moreno et al 1993). Furthermore, a decrease in G10H activity was demonstrated on elicitation (Moreno et al 1996). A lack of induction of G10H transcription was also reported in the initial report of ORCA3 cloning (van der Fitis & Memelink 2000). Simply, it isn’t clear that elicitation always induces increased alkaloid accumulation or that specific transcription factors elicit all necessary genes for increased alkaloid accumulation. Another concern is that elicitation may activate catabolic pathways that break down the indole alkaloids. In fact, two elicitation studies point to an increase in catabolic activity and the turnover of specific indole alkaloids (Moreno et al 1993; Rijhwani & Shanks 1998). A previous study on C. roseus hairy roots using jasmonic acid as the elicitor also provides valuable background (Rijhwani & Shanks 1998). While ajmalicine, serpentine, and hörhammericine increased continuously, tabersonine and lochnericine went through a maximum and then decreased below control levels. Some of the effects of elicitation were transients that might have been missed in this experiment by evaluation of alkaloid accumulation at only the six-day timepoint. In addition to checking for transient effects of induction, a few other studies could be pursued which would help characterize the behavior of EHIORCA3-4-1. The activation of genes reported to be induced by ORCA3
could be tested by northern analysis or enzyme assays to confirm that the ORCA3 expressed here is active. The effects of induction could also be compared with the effects of jasmonic acid elicitation.

7.3 Conclusion

Metabolic engineering of *C. roseus* for improved alkaloid yields will likely require the simultaneous overexpression of multiple genes and successful improvement of terpenoid pools. While the results presented here leave a number of questions unanswered, they do represent significant first steps in evaluating the available means for successful engineering of alkaloid yields. Certain positive signals are evident in the G10H and DXS efforts, and the ORCA3 study points out potential problems with the transcriptional activator strategy for increasing alkaloid yields.

7.4 Materials and Methods

7.4.1 Cloning of Genes and Plasmid Construction

ORCA3 was amplified from genomic DNA through standard PCR technique and sequenced for verification. The DXS gene used in this study is from *Arabidopsis*, and Dr. Patricia Leon generously provided a cDNA clone. G10H was amplified from cDNA prepared with a polyT primer, RNA purified from *C. roseus* hairy roots, and M-MLV reverse transcriptase according to manufacturer’s instructions (Promega). The resulting gene was sequenced and identically matched the published sequence. The DXS gene was supplied in pBluescript, while ORCA3 and G10H were moved from pCR2.1-TOPO
(Invitrogen) into pBluescript as EcoRV/SpeI fragments. Each gene was then removed as a XhoI/SpeI fragment and ligated into the glucocorticoid inducible plasmid pTA7002 to form p7002ORCA3, p7002DXS, and p7002G10H.

7.4.2 Clone Generation

The plasmids described above were electroporated into *A. rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L Yeast Extract, 0.2 g/L NaCl, 0.2 g/L MgSO₄·7H₂O, 4 mg/L FeCl₃, 0.66 g/L K₂HPO₄·3H₂O, 10 g/L mannitol, 15 g/L agar) including kannamycin (50 mg/L) were analyzed for the presence of the respective plasmid by restriction digest. A single colony was then used to start a 5 mL culture grown at 28 °C and 200 rpm for 36 hours in liquid YEM + kannamycin (50 mg/L) media. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described. After six weeks, hairy roots were excised from the plants and selected on solid and liquid media as described except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest.

7.4.3 Culture Conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose, half strength Gamborg’s B5 salts, and full strength vitamins (pH 5.7). Cultures were initiated by placing five tips into a 250 Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Induction was performed by adding the
appropriate volume of a 5 mM dexamethasone stock in ethanol. An equal amount of ethanol was added to uninduced controls.

7.4.4 RNA Isolation and Northern Analysis

Hairy root flask cultures that were approximately four weeks old were used for transcriptional induction studies. A 5 mM stock solution of dexamethasone was used to induce each cell line with ethanol added to the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen) according to manufacturer’s instructions. RNA was quantified by spectrophotometer according to absorbance at 260 and an ethidium bromide stained test gel used to verify the quality of RNA. 12 µg of total RNA were loaded per well and northerns performed essentially according to a reference protocol (Brown & Mackey 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen QIAEX II kit. Probes were then made using Stratagene Prime-It II kit.

7.4.5 HPLC analysis

Harvested cultures were immediately frozen at –80 °C. After lyophilization, a crude MeOH extract was prepared by extracting 50 mg of freeze-dried biomass with 10 mL of MeOH for an hour in a sonicating bath. Extracts were clarified at 1300g for 15 min. at 15 °C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then
passed through a 0.22 μm Nylon filter (13 mm). 10 μL of this extract was injected onto a Phenomenex Luna 5μ C18(2) HPLC column (250x4.6 mm). The Thermo Separations Products Spectrasystem HPLC system used included a P4000 pump unit, an AS3000 autosampler, and a UV2000 detector. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericicine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described. For the first five minutes, the mobile phase consisted of a 20:80 mixture of 50% MeOH/50% MeCN : 5 mM (NH₄)₂HPO₄ pH 6 pumped at 1 mL/min. Over ten minutes, it was linearly ramped to a 64:36 mixture where it was maintained for fifteen minutes. Over the next five minutes, the flow rate was linearly ramped to 1.4 mL/min. The ratio was then increased to 80:20 over five minutes where it was maintained for 15 minutes.

7.4.6 LCMS

LCMS analysis was performed at the Mass Spectrometry Consortium for the Life Sciences facility at the University of Minnesota. The positive ion electrospray mass spectra were obtained using the Phenomenex Luna 5μ C18(2) HPLC column (250x4.6 mm) with a Thermofinnigan LCQ ion trap mass spectrometer and a 20 μL injection. The system was also outfitted with a TSP UV6000 for PDA analysis. Two solvents at a flow rate of 0.7 mL/min were used: A (95% H2O/ 5% MeCN/ 0.2% acetic acid) and B (MeCN/0.2 % acetic acid). From 0 to 10 minutes, the ratio was 84:16. From 10 to 25 minutes, it was changed to 62:38. From 25 to 40 minutes, it was linearly changed to
10:90 where it was maintained for 20 minutes. The conditions were returned to the starting ratio and the column equilibrated. 150 µL/min were sent to the MS for analysis.

7.5 Acknowledgements

The authors would like to thank Dr. Nam-Hai Chua at the Rockefeller University for providing the inducible promoter plasmid (pTA7002), Dr. Eugene Nester at the University of Washington for providing the A. rhizogenes 15834 strain used, Dr. Johan Memelink at the University of Leiden for providing us with the geraniol 10-hydroxylase sequence prior to publication, and Dr. Patricia Leon from the Universidad Nacional Autonoma de Mexico for providing the cDNA clone of Arabidopsis DXS. This work was supported by funding from the National Science Foundation (Grant Numbers BES-0003730, BES-9906978). Erik Hughes was supported by a training grant from the National Institutes of Health (T32-GM08362).
Chapter 8

Preliminary Results from Inducible Expression
of Tabersonine 16-Hydroxylase

8.1 Introduction

*Catharanthus roseus* has warranted significant study due to its production of the valuable chemotherapeutics vincristine and vinblastine. Unfortunately, neither appears to be produced in cell suspension culture or hairy root culture. While catharanthine which provides half of the bisindole structure to vincristine and vinblastine is produced in these systems, the other half vindoline is not produced at any appreciable level. This inactivity has at least been partially explained by developmental and environmental requirements for the production of vindoline. Previous reports indicate that the six step pathway from tabersonine to vindoline requires light (De Carolis et al 1990; DeLuca et al 1989a; DeLuca & St.-Pierre 2000; St.-Pierre et al 1998) and that two late enzymes in this pathway show activity only in particular specialized cells of the stem and leaves (St.-Pierre et al 1999). Fortunately, metabolic engineering does provide us with the ability to overcome some native regulatory controls.

Tabersonine and two derivative compounds, lochnericine and hörhammericine, are prevalent alkaloids in *C. roseus* hairy roots. While genes for only three of the six steps between tabersonine and vindoline have been cloned, a recent study reported cloning of the first step, tabersonine 16-hydroxylase (T16H) (Schroder et al 1999). The other two cloned genes, desacetoxyvindoline 4-hydroxylase (D4H) (Vazquez-Flota et al
1997) and deacetylvinodoline acetyltransferase (DAT) (St.-Pierre et al 1998) represent the final two steps of the conversion (Figure 8-1). The availability of the first gene, T16H, is vital because it enables researchers to begin metabolic engineering efforts for the production of vindoline. Particularly, it allows us to study the effects of overexpression on the redistribution of tabersonine from undesirable products like lochnericine and hörhammericine to more desirable precursors of vindoline. By allowing for rechanneling of flux at this node, such studies can also possibly provide insight on the activity of the uncloned steps immediately following T16H.

T16H provides a valuable tool for the metabolic engineering of a tissue culture system known to accumulate high levels of tabersonine and derivatives but not vindoline. While overexpression of this gene is unlikely to yield vindoline due to the strict regulation of other genes in the pathway, it represents a significant first step towards the engineering of vindoline production. In this short note, we report the cloning of the 5’ end of the T16H gene through Tail PCR, the expression of the full coding sequence behind a glucocorticoid inducible promoter in hairy roots, and a preliminary investigation on the metabolic effects of T16H induction.
Figure 8-1. Biosynthetic pathway from tabersonine to vindoline. T16H, tabersonine 16-hydroxylase; OMT, O-methyltransferase; NMT, N-methyltransferase; D4H, 4-hydroxylase; DAT, acetyltransferase.
8.2 Results and Discussion

8.2.1 Sequence Report

The paper that reported the initial cloning of T16H commented that no full clone was ever attained despite their efforts (Schroder et al 1999). As only a few base pairs at the 5' end were missing, they simply designed a suitable anchor for expression and functional characterization in E. coli. Within this work, we were interested in expressing the full coding sequence in a tissue culture system of the native plant and therefore attempted to clone the 5' end of the native gene. This was accomplished using three internal nested primers, a genomic DNA preparation, and Tail PCR as reported in the methods section. A clone of the desired length yielded sequence data that matched the published sequence in the overlapping region and contained new sequence data at the 5' end. The relevant portion of this sequence is reported in Figure 8-2.

8.2.2 Generation and Induction of Clones

A full-length copy of the T16H gene was inserted into the glucocorticoid inducible promoter plasmid and subsequently used for Agrobacterium rhizogenes infection of C. roseus plants. While three hairy root clones grew successfully on selection media and adapted successfully to liquid media, only one line, EHIT16H-34-1, was chosen for further study due to its fast growth rate and visible induction on northern analysis. The northerns for that line and the negative control line, EHINC-12-1, are shown in Figure 8-3 in both the uninduced and induced states. Induction was performed for 72 hrs at 3 µM dexamethasone, and clones were tested for expression of the inducible
GGTCGAGAGAGAAACACATCCCTCCAATAAGT
CAAAAAATAATTGATGTTCCGAAAAAAATTAAAA
ATTGCAAAAAAGCAACATATGGACCAAGACAA
GTGAAGGAATTTATAGAGACTCCCCTATAAAAGC
CATGCATCGTAAGGGCTTTTATCCTAAAGCACA
TCAATAACAAGCATTTCATCTCATGGAATT
CTATTATTTTCTCTACTTGGCCTTCTTTTTTT
CTGCTTATTTTATCAAAAACCACAAAAGAAATT

Figure 8-2. Sequence Data for 5’ end of T16H gene. Underlined sequence is new sequence additional to the previous report (Schroder et al 1999) and the bolded ATG represents the likely start codon.
Figure 8-3. Northern analysis of lines EHINC-12-1 and EHIT16H-34-1. Lines were induced (I) at 3 µM for 72 hours and RNA prepared with ethanol similarly added to uninduced (U) controls. 12 µg RNA was loaded and blotting performed. Probes were made for T16H, the glucocorticoid transcription factor (GVG), and Ubiquitin 3 (UBQ3).
transcription factor (GVG) and T16H. Ubiquitin 3 (UBQ3) from *Arabidopsis* served as the loading control. In the northerns, both the negative control and T16H line are shown to transcribe GVG constitutively in both the uninduced and induced states. Significant T16H transcription is noted only in the lane corresponding to the induced T16H clone. As previously reported in the original cloning report, low level transcription of a gene running at a slightly larger size was reported in all lanes including those of the negative control. Previous researchers thought this was a related gene of a different function due to the absence of T16H activity. This is a likely explanation, and the partial cloning of a cDNA clone with high homology is reported in Appendix A.

### 8.2.3 Metabolic Effects of Induction

If induced transcription of T16H results in increased diversion of tabersonine into the vindoline pathway, yields of tabersonine-related alkaloids including hörhammericicine and lochnericine should decrease. In order to test the effects of T16H induction, cultures in triplicate were induced at 3 μM dexamethasone for six days during the exponential growth phase. An equal amount of ethanol was added to uninduced controls. The yields, standard deviations, and percentage change of the specific alkaloid yields are shown in Table 8-1. As the effects of induction on the control line are minimal during the exponential phase as previously reported, they have been omitted. Tabersonine, lochnericine, and hörhammericicine decrease significantly by 75, 48, and 39 percent, respectively. If tabersonine is being converted into a competitive product, such decreases should be expected and are an encouraging sign. It is also encouraging that no
significant effects were noted for the Coryanthe alkaloids serpentine and ajmalicine as the pathways to the Aspidosperma and Coryanthe alkaloids diverge upstream of T16H.

From previous reports that studied the occurrence of various alkaloids and enzymatic activities, the accumulation of certain alkaloids along the pathway from tabersonine to vindoline might be expected. A study of native *C. roseus* roots indicated that roots accumulated only 16-hydroxytabersonine and that other products along the pathway were not present (DeLuca et al. 1986). Other relevant studies have also been performed on cell suspension cultures. They have indicated that NMT (DeLuca et al. 1987), D4H (Vazquez-Flota et al. 1997), and DAT (DeLuca et al. 1987) activities are not present. Even so, T16H and OMT activities for catalyzing the first two steps (St.-Pierre & DeLuca 1995) and activities for enzymes that couple vindoline and catharanthine were found (Endo et al. 1988).

As any direct product of tabersonine including 16-hydroxytabersonine and 16-methoxytabersonine will share the \( \alpha \)-methyleneindole structure, initial evaluation was done using the chromatogram at the \( \lambda_{\text{max}} \) of this chromophore, 329 nm. When comparing the chromatograms from induced and uninduced cultures (Figure 8-4), the most obvious difference is the substantial peak at 19 minutes. The decrease in tabersonine, lochnericine, and hörhammericine yields is also apparent. PDA analysis of the unknown peak confirmed a spectra similar to a tabersonine standard with a shifted maxima (\( \lambda_{\text{max}}=336 \) nm) but further identification was not successful. However, LCMS efforts to identify a peak that appeared on induction yielded interesting results. The UV spectrum for the peak is compared with that of a tabersonine standard in Figure 8-5. As
other peaks visible at 329 elute at very similar retention times, there is likely some interference in the unknown spectrum. Fortunately, LCMSMS data provides some convincing data for identification of the new peak. The original LCMS spectrum of this peak and the LCMSMS fragmentation pattern are shown in Figure 8-6. The m/z of the original molecule is 367. On fragmentation, the highest intensity fragments were 335 and 258. Additional lower-intensity fragments included 307, 279, and 136. The original mass and the mass of the fragments noted is consistent with previously reported data for 16-methoxytabersonine (Schroder et al 1999). The peak also appears only on induction and has a retention time very similar to tabersonine as expected (J. Schröder, personal communication). Therefore, it seems that induced overexpression of T16H results in the synthesis of 16-methoxytabersonine. The discovery of this alkaloid is the first report to the author’s knowledge of this metabolite in native or hairy roots. Furthermore, previous efforts by our laboratory have not identified any metabolite along the pathway from tabersonine to vindoline in other hairy root lines (Shanks et al 1998). The existence of 16-methoxytabersonine not only demonstrates that some flux was directed towards vindoline by inducible expression of T16H but that some native OMT activity exists in hairy roots for the conversion of 16-hydroxytabersonine to 16-methoxytabersonine. The diminished yields of tabersonine, lochnericine, and hörhammericicine are also consistent with the indication that flux has been successfully diverted at the tabersonine branch point. While purification and subsequent identification of unknown peaks would allow further characterization of T16H’s role in hairy roots, the glucocorticoid-induced synthesis of 16-methoxytabersonine alone is a significant result.
<table>
<thead>
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<th>Alkaloid</th>
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<th>% Change</th>
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</tr>
<tr>
<td>Serpentine</td>
<td>611</td>
<td>51</td>
<td>532</td>
</tr>
</tbody>
</table>

**Table 8-1.** Alkaloid analysis for line EHI16H-34-1. Cultures were induced in triplicate for six days at 3 µM with ethanol added to the uninduced controls. * indicates p<0.05.
Figure 8-4. Chromatograms of the uninduced and induced extracts from transgenic T16H line at 329 nm. Cultures were induced for six days, and crude methanol extracts prepared. Peak 1 represents hörhammericine, peak 2 represents lochnericine, peak 3 represents tabersonine, and peak 4 represents an unknown peak.
Figure 8-5. PDA spectra of tabersonine (A) and unknown peak that appears on induction (B). Tabersonine elutes at 20 minutes with unknown at 21 minutes.
Figure 8-6. MS data for unknown peak visible on induction. (A) is the original LCMS spectrum of the peak and (B) is the LCMSMS fragmentation pattern.
8.3 Conclusions

The tabersonine to vindoline pathway is a highly regulated pathway that has been extensively studied. Currently, it is the known barrier to vindoline, vincristine, and vinblastine production in cell suspension and hairy root cultures. Three of the six necessary genes are currently cloned, and continuing efforts may enable the engineering of the entire pathway to vindoline. Here, we report the full coding sequence of T16H, the generation of a hairy root line transgenic for inducible T16H expression, and the glucocorticoid-induced production of 16-methoxytabersonine indicating activity along the desired pathway.

8.4 Materials and Methods

8.4.1 Tail PCR

Tail PCR was performed by adaptation of a previously reported method (Liu et al 1995). In addition to the three random primers described in the reference, three nested primers for tabersonine 16-hydroxylase from the published sequence were synthesized. These three primers were T16HTAIL1 (GGGCTTTGATTTTCCTCTTA), T16HTAIL2 (GCACGCCTCTTACATTTCCATAAA), and T16HTAIL3 (TTGAGGTTCTATCGGCAAAAA). The PCR reaction was performed against previously purified genomic DNA from C. roseus and showed a product of the desired length with random primer AD2 (NGTCGASWGANAWGAA, N=A,C,G,T; S=C,G; W=A,T). Briefly, a PCR reaction as previously reported is performed with AD2 and T16HTAIL1. The product is diluted and used for a secondary reaction with AD2 and T16HTAIL2.
Repetition again with T16HTAIL3 for the tertiary reaction yielded a band that was sequenced for verification.

8.4.2 Plasmid Construction

The native tabersonine 16-hydroxylase gene contains a VspI site within the gene which was used for cloning purposes. The gene likely contains introns and initial attempts at genomic PCR were unsuccessful. Using a total RNA preparation from seedlings which were light induced for 2 days, cDNA was prepared using M-MLV reverse transcriptase with primer T16HLONGBW (GAATTGTTTCGGTAAAGAT TTTCAAGCAGGAGAAAGAGC) according to manufacturer’s instructions (Promega). A PCR was then performed against this cDNA to amplify the 3’ end of the gene. The 5’ end as previously determined was amplified from genomic DNA. The 3’ end of T16H was cut with VspI/SpeI and the 5’ end was cut with XhoI/VspI for ligation into SpeI/XhoI cut pBluescript. After successfully generating a resistant clone, the gene was fully sequenced and subsequently moved as a XhoI/SpeI fragment into the glucocorticoid plasmid pTA7002 to form p7002T16H.

8.4.3 Clone Generation

The plasmid described above, p7002T16H, was electroporated into *A. rhizogenes* 15834. Transformed colonies from YEM plates (1 g/L Yeast Extract, 0.2 g/L NaCl, 0.2 g/L MgSO₄•7H₂O, 4 mg/L FeCl₃, 0.66 g/L K₂HPO₄•3H₂O, 10 g/L mannitol, 15 g/L agar) including kannamycin (50 mg/L) were analyzed for the presence of the respective
plasmid by restriction digest. A single colony was then used to start a 5 mL culture grown at 28 °C and 200 rpm for 36 hours in liquid YEM + kanamycin (50 mg/L) media. Scissors were dipped in the culture and used to infect aseptically grown plants as previously described. After six weeks, hairy roots were excised from the plants and selected on solid and liquid media as described except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest.

8.4.4 Culture Conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose, half strength Gamborg’s B5 salts, and full strength vitamins (pH 5.7). Cultures were initiated by placing five tips into a 250 Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Induction was performed by adding the appropriate volume of a 5 mM dexamethasone stock in ethanol. An equal amount of ethanol was added to uninduced controls.

8.4.5 RNA isolation and Northern Analysis

Hairy root flask cultures that were approximately three weeks old were used for transcriptional induction studies. A 5 mM stock solution of dexamethasone was used to induce each cell line with ethanol added to the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen) according to manufacturer’s instructions. RNA was quantified by spectrophotometer according to absorbance at 260 and an ethidium bromide stained test gel used to verify the quality of
RNA. 12 μg of total RNA were loaded per well and northerns performed essentially according to a reference protocol (Brown & Mackey 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen QIAEX II kit. Probes were then made using Stratagene Prime-It II kit.

8.4.6 HPLC Analysis

Harvested cultures were immediately frozen at −80 °C. After lyophilization, a crude MeOH extract was prepared by extracting 50 mg of freeze-dried biomass with 10 mL of MeOH for an hour in a sonicating bath. Extracts were clarified at 1300g for 15 min. at 15 °C. The supernatant was removed and the biomass reextracted in the same manner. Extracts were combined and concentrated under vacuum to 2 mL and then passed through a 0.22 μm Nylon filter (13 mm). 10 μL of this extract was injected onto a Phenomenex Luna 5μ C18(2) HPLC column (250×4.6 mm). The Thermo Separations Products Spectrasystem HPLC system used included a P4000 pump unit, an AS3000 autosampler, and a UV2000 detector. Ajmalicine and serpentine were detected at 254 nm and quantified by comparison with authentic standard curves. Tabersonine, lochnericine, and hörhammericine were detected at 329 nm by retention time of standards and quantified based on a tabersonine standard curve as previously described. For the first five minutes, the mobile phase consisted of a 20:80 mixture of 50% MeOH/50% MeCN : 5 mM (NH₄)₂HPO₄ pH 6 pumped at 1 mL/min. Over ten minutes, it was linearly ramped to a 64:36 mixture where it was maintained for fifteen minutes. Over the next five
minutes, the flow rate was linearly ramped to 1.4 mL/min. The ratio was then increased to 80:20 over five minutes where it was maintained for 15 minutes.

8.4.7 LCMS

LCMS analysis was performed at the Mass Spectrometry Consortium for the Life Sciences facility at the University of Minnesota. The positive ion electrospray mass spectra were obtained using the Phenomenex Luna 5\(\mu\) C18(2) HPLC column (250x4.6 mm) with a Thermofinnigan LCQ ion trap mass spectrometer and a 20 \(\mu\)L injection. The system was also outfitted with a TSP UV6000 for PDA analysis. Two solvents at a flow rate of 0.7 mL/min were used: A (95% H2O/ 5% MeCN/ 0.2% acetic acid) and B (MeCN/0.2 % acetic acid). From 0 to 10 minutes, the ratio was 84:16. From 10 to 25 minutes, it was changed to 62:38. From 25 to 40 minutes, it was linearly changed to 10:90 where it was maintained for 20 minutes. The conditions were returned to the starting ratio and the column equilibrated. 150 \(\mu\)L/min were sent to the MS for analysis.

8.5 Acknowledgements

The authors would like to thank Dr. Nam-Hai Chua at the Rockefeller University for providing the inducible promoter plasmid (pTA7002) and Dr. Eugene Nester at the University of Washington for providing the \textit{A. rhizogenes} 15834 strain used. Thomas Krick provided valuable help with LCMSMS. This work was supported by funding from the National Science Foundation (Grant Numbers BES-0003730, BES-9906978). Erik
Hughes was supported by a training grant from the National Institutes of Health (T32-GM08362).
Chapter 9

Conclusions and Future Directions

There are a number of positive outcomes presented in the results of the preceding thesis. An inducible promoter was tested and found to be active within *C. roseus* hairy roots. Combined with the ability to generate transgenic hairy roots, this advance should enable improved metabolic engineering studies and data interpretation for metabolites that exhibit high clonal variation. From a base that included transgenic studies using only three relevant alkaloid genes in cell culture, this thesis including preliminary results has expanded those efforts in an advantageous system to the indole pathway, the terpenoid pathway, and a valuable alkaloid pathway. In the indole pathway, tryptophan and tryptamine yields were dramatically increased by feedback-resistant anthranilate synthase induction. With inducible tryptophan decarboxylase activity alone, serpentine specific yields increased. A feeding study in the terpenoid pathway demonstrated the importance of the deoxyxylulose pathway and the potential increases in alkaloids that could be achieved with metabolic engineering. Preliminary results with inducible geraniol 10-hydroxylase (G10H) and deoxyxylulose 5-phosphate synthase (DXS) showed increased yields of particular alkaloids. Finally, a full-length clone of tabersonine 16-hydroxylase was reported and used to alter flux distribution at an important branchpoint within the alkaloid pathways resulting in a vindoline precursor. All the reported studies with one exception altered only one gene in the pathway, but greater success in engineering the alkaloid pathways will likely be dependent on engineering multiple steps and multiple pathways simultaneously.
An interesting starting point of future work would be the feeding of terpenoid precursors to lines known to oversynthesize indole precursors in order to check the need for coordinate regulation. Such studies have been pursued in cell culture and could easily be done on the AS line reported here that accumulated significant amounts of tryptophan and tryptamine. Gains are likely and would point to the need to engineer both pathways simultaneously. After the DXS and G10H lines are further characterized, indole precursors could also be fed to these lines. Characterization efforts could potentially involve peak identification by methods reported in an earlier group thesis (Vani 1996) and the development of methods for quantifying the terpenoid precursors. With an improved understanding of terpenoid metabolism, multigene efforts to simultaneously alter the indole and terpenoid branches could be pursued.

Another effort that could prove rewarding is to focus on understanding the production of indole alkaloids in *C. roseus* hairy roots at the molecular and enzymatic level. Other than a few isolated reports on strictosidine synthase and tryptophan decarboxylase, very few reports have focused on the activity, transcription, or regulation of numerous steps in hairy roots. While it is possible to depend on cell culture and whole plant studies for guidance, a focused molecular study in *C. roseus* hairy roots would be extremely valuable for future engineering efforts. Something as simple as studying the transcription of all cloned genes in standard culture and under elicitation could provide a valuable baseline. Another study that could prove interesting is to examine the transcription of various terpenoid genes when treated with carotenoid-inducers which previously increased alkaloid levels in cell culture (Lee et al. 1981).
In the disciplines of genetic cloning and regulatory characterization of alkaloid biosynthetic enzymes, there remain a few developments that would be vital to future progress. At the current time, substantial research effort is being focused on the terpenoid pathway in model plant systems such as tomato and *Arabidopsis*. If these efforts result in increased regulatory knowledge and the ability to substantially increase flux through the terpenoid pathway, the potential to beneficially alter the pathway for alkaloid production will be enabled just as it was for the AS study. Continued progress on the cloning of additional alkaloid genes particularly from tabersonine to vindoline would also prove vital. A recent report of such an attempt shows that there is continuing interest in this endeavor (Cacace et al. 2003). The work in this thesis has presented a method to increase indole flux but substantial effort is still warranted on individual methods to increase terpenoid flux and the accumulation of desired alkaloids.

The availability of several laboratory techniques would also prove valuable. I attempted both cryopreservation and regeneration in cursory studies during my Ph.D. and was unsuccessful. The failure to regenerate plants from *C. roseus* hairy roots is not surprising as all previous regeneration attempts for transgenic *C. roseus* have been unsuccessful. Methods exist for a variety of plant systems (Kaur et al. 1996; Kim et al. 1994; Mollers & Sarkar 1989; Momcilovic et al. 1997; Perez-Molphe-Balch & Ochoa-Alejo 1998; Tanaka et al. 1994; Zarate et al. 1999), and the availability of such a reproducible method for *C. roseus* would be a huge development. Another technique that would be valuable for continuing efforts in our laboratory is a method to cryopreserve hairy root lines. After exerting the significant effort required to create the
lines, they must still be maintained by sterile subculturing every month. This process is prone to contamination and a backup storage method would be helpful. Cryogenic methods do exist for hairy root line preservation and might warrant further study (Benson & Hamill 1991; Hirata et al 2002). Overall, there remain a number of directions and studies, which would contribute to the progress of plant biotechnology and particularly the study of *C. roseus*.

The preceding thesis was focused on the engineering of the indole pathway and the development of the inducible promoter as an effective tool. Immediate future efforts should complete the preliminary work presented here. Other potentially important contributions include establishing methods to engineer terpenoid flux and means to characterize altered terpenoid metabolism. Multigene studies and continued concentration on the vindoline pathway are also warranted. Although the pace of the progress will be largely dependent on simultaneous efforts by molecular biologists, metabolic engineering will play a vital role in applying any gained knowledge to the genetic improvement of the overall system.
Appendix A

Sequence of cDNA with High Homology to Tabersonine 16-Hydroxylase

When trying to PCR the 3’ end of the tabersonine 16-hydroxylase gene from a cDNA preparation as described in Chapter 8, a highly homologous DNA fragment was cloned into the plasmid p3prime4 and partially sequenced. The genetic sequence presented below is not represented in Genbank and is particularly interesting because it shows such high homology to tabersonine 16-hydroxylase over the three sections shown in Figure A-1. A previous genomic Southern blot from the original tabersonine 16-hydroxylase report indicated that C. roseus likely contained at least two closely related genes (Schroder et al 1999), and this is a likely suspect.

Plasmid p3prime4 Sequence Data, 661 bases

GAGGGTACAAGAATTTAATTCCAGCAAGGAATATCATTACTTTATATGTG
AATTACTACCCGCTGAGCTTTTTGGAGAAAAAAATAAAGGATACAGAAGAT
TTATTTCGCTCTTTTGTATCAACTCACAAGGCAACACGCGATCCTAACATT
GCCGATATGGTCCCTTCTATCAAATTTCTCTACTTTATAATTAGTAGTACAA
ATACAAGATGGAGATAATACACAATTTTTATGATGCCATAAGTCAAACAA
TTCTCAACCATTAGGAAGATCAGTCAATTGTGAAGTCATCTCAAGTCTAT
GAAGCAGATGGGGAACAAAACAAAGGACTTTTGGATGATGTGCTACTCAATAT
TCAAAACACGTGGTGTTTTGTATATTTACCACACTTAGGTGATCAACGGTGCAAG
CAGTAATCTTTTTGAGTACCTCAATTCTTTTATAATTATTTTATGTG
CACACGGAGTAGATATACACTGAAATCTCCATTTTTCTCCATTTTTTCCA
CTTTAAAATCTCTCAAAAACCTTTGATCTCTTTACTTTTGGTTTCAAGAACATATT
TGGTGCCCGAAGGACAATCTCAACCACAGTGTTGAGGCGATGTTGTG
AAATGATAAAAAATTCTCAAGATATGAAAAAGGCACAAGAAGGTAAGA
AGGTATTTCATGA
Figure A-1. Sequence alignment of three underlined sections in p3prime4 sequence with tabersonine 16-hydroxylase showing high homology.
Appendix B

Protein and Nonprotein Nitrogen in *Catharanthus roseus* Hairy Roots

The work presented in Chapters 4 and 5 within this thesis focuses on engineering flux through the indole pathway in *C. roseus* hairy roots. As tryptophan is converted into a number of plant metabolites including indole glucosinolates, auxins, protein, tryptamine, and indole alkaloids, a literature search was conducted to analyze the primary metabolic fates (Radowanski & Last 1995; Siehl 1999). Of those previously listed, only free tryptophan, tryptamine, indole alkaloids, and protein are expected to be present in significant amounts within our system (Baldi et al 1991; Biondi et al 1997; Haughn et al 1991). In order to gain a better understanding of the protein levels within our system, a preliminary study on the level of protein, protein nitrogen, and nonprotein nitrogen was conducted. The motivation in pursuing this topic was that in order to study the flux through tryptophan one would need to understand its accumulation in various end products. HPLC provides methods for measuring tryptophan, tryptamine, and specific indole alkaloids but no methods to determine total protein had been explored. While catabolism and turnover of metabolites are relevant concerns, their importance can be minimized by appropriately controlling the time scale of the experiment.

*C. roseus* is not an agriculturally important crop or a widely studied plant system and therefore no well developed method for estimating total protein exists. Certain methods like the Bradford and Lowry methods depend on chemical reactions with proteins but also require that the proteins are soluble and extractable (Bradford 1976; Lowry et al 1951). Other methods like Kjeldahl and combustion analysis provide
measures of nitrogen but require a conversion factor to protein that is dependent on the amino acid composition of total protein and the system’s levels of nonprotein nitrogen including free amino acids, alkaloids, DNA, RNA, and nitrates. Unfortunately, metabolic characterization studies are limited in our system and therefore conversion factors are not available. Originally, I had planned to perform a mass balance using labeled fed tryptophan in order to determine the relative importance of measurable metabolic fates within our system. A preliminary study was conducted on methods to determine protein levels but was never expanded beyond those results. That data is presented here as it might prove useful to mathematical flux analysis of our system or future tryptophan fate studies.

Our interest in measuring tryptophan metabolic fates was derived from our efforts to metabolically engineer the availability of tryptophan. In studying the protein levels, we therefore chose to study the effects of tryptophan feeding on the various tryptophan sinks. Tryptophan was fed at 52 μM on day 17 to clone LBE-6-1 in triplicate, and cultures were harvested at 17, 19, 21, 23, and 25 days in triplicate. No growth effects were noted until the 25 day timepoint at which fed cultures had higher DWs. After freeze-drying and homogenizing the cultures, 40 mg samples were subjected to various treatments and analysis. First, 40 mg of biomass was loaded into a LECO CHN 2000 combustion analyzer to determine total nitrogen. 40 mg was also extracted twice with 0.8 M TCA with subsequent cold acetone washing of the pellet. TCA makes most proteins insoluble and therefore allows soluble nonprotein nitrogen to be extracted. The
Figure B-1. Combustion analysis of biomass with nitrogen content measured as a percent of original DW mass. Total nitrogen was determined by combustion analysis of unaltered biomass. Protein nitrogen including unextractables was determined by combustion analysis of TCA extracted mass. Nonprotein nitrogen (NPN) is the difference. All data are means +/- standard deviations.
remaining solid pellet containing protein nitrogen and unextractable nonprotein nitrogen was freeze-dried and analyzed by combustion. Extractable nonprotein nitrogen is then the difference between the total nitrogen and the protein/unextractable fraction. The data from this nitrogen combustion analysis is presented in Figure B-1.

Protein levels were also explored through Bradford and Lowry analysis both in crude extracts and after TCA protein precipitation plus resolubilization. Here we will only present the Bradford assay results before precipitation as all methods reported similar trends. In assaying the crude extracts, the Lowry method appears to require 20% TCA precipitation due to an interfering substance. As previously noted, the spectrophotometric methods require a reaction to determine protein levels. In the Bradford assay, a negatively charged dye must bind to positive charges in the protein. The resulting absorbance is then converted to protein through the use of a standard curve generated using BSA or another representative protein as the standard. Using an average conversion factor of 6.25 g protein/g Nitrogen, these results could then be converted into nitrogen levels for comparison to previous data. In addition to the dependence on the representative protein and the conversion factor, the Bradford method is dependent on the ability to solubilize the protein. In this study, we extracted the sample twice with H₂O, twice with 0.5 M NaCl (pH 7.3), and twice with 0.1 M NaOH. To check the solubilization of the nitrogen content, the remaining pellet after extraction was subjected to combustion nitrogen analysis. Unfortunately, as much as 40% of the nitrogen in some samples remained unextracted. The data for the unextracted pellet and the Bradford extraction are presented in Figure B-2.
Figure B-2. Buffer extraction analysis of biomass with nitrogen content measured as a percent of original DW mass. Soluble protein nitrogen was determined by Bradford analysis. Unextracted nitrogen was determined by combustion analysis of the remaining pellet.
The total nitrogen can certainly be split into soluble nonprotein nitrogen, insoluble nonprotein nitrogen, soluble protein nitrogen, and insoluble protein nitrogen. The buffer extraction method measures soluble protein nitrogen by Bradford and both insoluble fractions as unextractables. The original combustion analysis measures soluble nonprotein nitrogen as one fraction and both protein fractions and insoluble nonprotein nitrogen as the other. By combining the protein nitrogen from the Bradford analysis with the unextractable nitrogen from the Bradford assay, the buffer extraction results can be reconciled with the combustion analysis of protein nitrogen as reported in Figure B-3. Unfortunately, insoluble nonprotein nitrogen and insoluble protein content cannot be resolved. A feeding study using labeled tryptophan would prove useful in determining the relevance of these insoluble fractions to the determination of tryptophan flux.

The study does provide us with some useful information in addition to lower and upper bounds on protein nitrogen content. Tryptophan feeding has very little effect on the protein levels or any other measured variable. Soluble nonprotein nitrogen varies from 54% to 35% of total nitrogen indicating that it must be considered in any method which utilizes nitrogen levels for the determination of protein levels. If one assumes protein is 1.6% tryptophan as determined in another study (Dewanji 1993) and that the DW biomass measured is 7.5% protein, it is also possible to gain an understanding of the allocation of tryptophan in *C. roseus* hairy roots. Using the fact that the line used in this study maintains a relatively constant 8 mg/g DW of individually assayed indole alkaloids, three times as much tryptophan is accumulated as indole alkaloids than as proteins. Further analysis of the results reported here would require a specific
Figure B-3. Comparison of buffer extraction results with combustion analysis of TCA extracted biomass with nitrogen content measured as a percent of original DW mass. Both data sets now represent unextractable nitrogen and soluble protein. Unextractable nonprotein nitrogen and unextractable protein nitrogen still cannot be separated.
determination of the tryptophan content of *C. roseus* proteins and labeled work to determine the relevance of the nonsoluble fractions.
References


tobacco hairy root cultures by its fusion to a rbcS transit peptide coding sequence. *Transgenic Res.* 5:193-201.


