Genetic engineering of *Catharanthus roseus* to enhance production of anticancer pharmaceutical compounds

A Thesis Presented

By

Noreen F. Rizvi

To

The Department of Chemical Engineering

In partial fulfillment of the requirements
For the degree of

Doctor of Philosophy

In the field of

Chemical Engineering

Northeastern University
Boston, Massachusetts

April 17, 2015
Acknowledgements

This thesis would not have been possible without the help of many people. First, I would like to express my deepest appreciation to my advisor, Dr. Carolyn Lee-Parsons, who has been such a dedicated and involved mentor. Her encouragement and enthusiasm for research and teaching has propelled my own pursuit of an academic career. She has always been available for my (many!) questions and has always led me in the right direction, which has been extremely helpful in achieving this doctorate.

I would also like to thank my co-advisor, Dr. Erin Cram (Dept. of Biology). Her guidance and genius has been invaluable to this very interdisciplinary project, which would not have been possible without her willingness to collaborate, teach, and serve as my co-mentor. I would also like to thank my other committee members, Dr. Anand Asthagiri and Dr. John Celenza (Dept. of Biology, Boston University), for their feedback, patience and time.

I truly appreciate the support of my fellow lab members, past and present, including graduate students: Sheba Goklany, Lutfiye Kurt, John Oldham, Jessica Weaver, Sydney Shaw, and John de la Parra, and undergraduate students: Kassi Stein, Miglia Cornejo, Caroline Webb, Ashley Allerheiligen, Jennifer Langh, and Melanie Guerin. It has been a treat working with you all and learning together in our lengthy lab discussions.

Immeasurable thanks to my family and friends, who have provided me with such strong support throughout these five years. Specifically, I’d like to thank: my parents and especially my father, Dr. Syed Rizvi, who has served as (yet another) academic mentor and life coach; my brothers; my fiancé, Zachary Strasser, who has encouraged me to never give up; and all my friends who have made Boston feel like home.

Lastly, I am thankful to the National Science Foundation (NSF) and the American Associate for University Women (AAUW) for funding, and the Department of Chemical Engineering at Northeastern University.
Abstract

The medicinal plant, *Catharanthus roseus*, is the source of pharmaceutically valuable anticancer alkaloids, vincristine (VCR) and vinblastine (VBL). These alkaloids are produced only in *C. roseus* and at extremely low levels (0.0002 wt%). Due to their low levels, the isolation of these compounds is both laborious and costly, ranging from $4 to $60 million/kg. Despite these barriers, these alkaloids have been effectively used to treat cancer for over 50 years.

The biosynthesis of VCR and VBL is not entirely known but involves at least 35 intermediates and 30 enzymes. In an effort to increase alkaloid production, key enzymes in the *C. roseus* biosynthetic pathway have been overexpressed but this strategy has proven unsuccessful since the enzymatic bottleneck is shifted downstream. However, transcription factors regulate multiple genes in the pathway and therefore engineering their expression may potentially increase the overall flux through the network and lead to increased production of the desired compounds. Therefore, in this thesis we investigate key transcription factors involved in regulating multiple alkaloid biosynthetic enzymes with the overall goal of improving alkaloid production to make these drugs cost-effective and available to patients.

First, we establish an optimized and efficient *Agrobacterium*-mediated method for the genetic transformation of *C. roseus* hairy root cultures, including a novel, estrogen-inducible construct for transgene expression (Chapter 4). Since ZCT1 (Zinc finger *Catharanthus* transcription factor) is a known repressor of alkaloid biosynthetic enzymes, we used the optimized transformation method and inducible construct to create transgenic *C. roseus* hairy roots with silenced Zct1 expression (Chapter 5). The results of silencing this key repressor and implications in alkaloid biosynthesis in *C. roseus* were analyzed. In addition to silencing Zct1 in hairy roots, Zct1 expression was also silenced transiently in *C. roseus* seedlings,
uncovering ZCT1’s role in growth (in addition to its existing role in defense, i.e. alkaloid production). ZCT1’s involvement in the mechanism by which growth and defense are compromised in *C. roseus* is studied (Chapter 6). This tradeoff between growth and defense in plants is poorly understood; uncovering the mechanism by which plants switch between these processes may lead to novel methods to overcome this compromise to ultimately increase yields and reduce costs of important, plant-derived, pharmaceutical compounds.
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1.0 Introduction

It is estimated that 25% to 50% of pharmaceuticals in the Western world are plant derived, with an even greater percentage in Asia and other parts of the world (Shuler 2002). The diversity of the plant kingdom suggests that there is an untapped library of structurally diverse compounds for improving human health and treating diseases. *Catharanthus roseus* produces over 130 different terpenoid indole alkaloids (TIAs) including vincristine (VCR) and vinblastine (VBL), two powerful anticancer drugs (Figure 1.1). These TIAs are only produced by the plant in a defense or stress response, such as pathogen attack or wounding. As a result, they are produced at extremely low levels, resulting in their costly ($4 – 60 million/kg) and laborious isolation (van der Heijden et al. 2004). Furthermore, the complex structure of these alkaloids makes their chemical synthesis difficult and thus far unachievable.

![Chemical structure of vincristine and vinblastine](image)

**Figure 1.1: Anticancer alkaloids, vincristine and vinblastine, produced in C. roseus**

Despite barriers, these compounds have been used to treat cancer effectively for over 40 years due to their unique mode of action and their effectiveness. For these reasons, VCR and VBL are excellent targets for genetic engineering to increase their production, meet their demand, and decrease their cost. There has been tremendous advancement in the understanding of the complex biosynthetic pathway that produces these drugs. The biosynthesis of VCR and VBL is
complex, involving at least 35 chemical intermediates, 30 enzymes, and 7 intra- and intercellular compartments (van der Heijden et al. 2004). Previous attempts to engineer the overexpression of specific key enzymes in the pathway have been largely unsuccessful; while the production of nearby intermediates increases, the bottlenecks to TIA production simply shift downstream and TIA production does not increase.

Since transcription factors simultaneously regulate multiple key enzymes in the pathway, engineering their expression may increase the overall flux through the network and lead to increased production of the alkaloids. Jasmonate (JA; or methyl jasmonate, MJ), a plant hormone produced in a defense response, activates a cascade of transcription factors which control alkaloid production. My research focuses on investigating and genetically manipulating these transcription factors. Our goal is to control and optimize TIA production by *C. roseus* hairy root cultures through understanding and controlling the transcription factors that regulate TIA production.
2.0 Critical Literature Review

2.1. *Catharanthus roseus* and the pharmacological activity of its TIAs

The *Catharanthus* genus is a part of the Apocynaceae family, and *Catharanthus roseus* (commonly known as the Madagascar periwinkle) is a short shrub with stems ranging between 3-100 cm in length (Figure 2.2) (Memelink and Gantet 2007; van der Heijden et al. 2004). *C. roseus* is a native species to the Indian Ocean island of Madagascar and is commonly found in tropical or sub-tropical climates. *C. roseus* is a chemical factory, producing over 130 different terpenoid indole alkaloids (TIAs) as secondary metabolites. Many of these TIAs are pharmacetically important, making *C. roseus* a widely coveted and studied species.

![Catharanthus roseus plant and the structure of various TIAs it produces](image)

**Figure 2.2: Catharanthus roseus plant and the structure of various TIAs it produces**

Two important TIAs, vinblastine (VBL) and vincristine (VCR) (Figure 2.2), have been used as anticancer drugs for the last 50 years due to their effectiveness and mode of action. VBL and VCR specifically target microtubule action in cells, ultimately causing apoptosis, especially in rapidly dividing cancer cells. VBL, discovered in 1960, is currently used in the treatment of Hodgkin’s and non-Hodgkin lymphomas, testiscarcinomas, breast cancer, and chorio-carcinomas.
VCR, an oxidized form of VBL and discovered in 1963, is used to treat acute leukemia, Hodgkin’s and non-Hodgkin lymphomas, rhabdomyosarcomas, Wilm’s tumors in children, and breast cancer (van der Heijden et al. 2004).

Many other TIAs produced in *C. roseus* have pharmaceutical value. For example, yohimbine can have an antiviral effect on herpes simplex virus (type 1) and antifungal activity (Briones-Martín-Del-Campo et al. 2014; Özçelik et al. 2011). Vindoline, vindolidine, vindolicine and vindolinine from *C. roseus* increase glucose absorption in pancreatic cells and can be used to treat diabetes (Tiong et al. 2013). Ajmalicine (Figure 2.2), introduced in 1957, is used for the treatment of hypertension, and serpentine (Figure 2.2) is used as a sedative and also to treat hypertension (van der Heijden et al. 2004).

### 2.2. The biosynthesis of TIAs in *C. roseus*

The biosynthesis of TIAs such as VCR and VBL in *C. roseus* is very complex, involving over 35 intermediates, 30 enzymes, and 7 intra- and intercellular compartments (van der Heijden et al. 2004). TIAs are condensation products from two separate pathways: the terpenoid pathway and the indole pathway (Figure 2.3) (van der Heijden et al. 2004). Secologanin (derived from the terpenoid pathway) and tryptamine (derived from the indole pathway) condense to form strictosidine, the backbone for the formation of all TIAs (Memelink and Gantet 2007; van der Heijden et al. 2004).

In the monoterpenoid pathway, geraniol is converted to 10-hydroxygeraniol by the enzyme geraniol 10-hydroxylase (G10H) (Memelink and Gantet 2007). In the indole pathway, tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC). Both of these steps are the first committed steps towards TIA biosynthesis in their respective pathways. Loganin is further converted to secologanin by the enzyme secologanin synthase (SLS) (Memelink and
Gantet 2007). Condensation of secologanin and tryptamine to strictosidine is catalyzed by strictosidine synthase (STR). Strictosidine is converted to cathenamine by the enzyme strictosidine β-D-glucosidase (SGD), which is further converted to TIAs ajmalicine, tabersonine, and catharanthine. Vinblastine and vincristine are further downstream TIAs, produced by the coupling of catharanthine and vindoline.
Figure 2.3: Terpenoid indole TIA (TIA) biosynthesis in *C. roseus*, including enzymes (in blue). Dashed lines indicate several enzymatic steps. DXS = 1-deoxy-D-xylulose-synthase; G10H = geraniol-10-hydroxylase; SLS= secologanin synthase; AS = anthranilate synthase; TDC = tryptophan decarboxylase; STR= strictosidine synthase; SGD= strictosidine β-D-glucosidase; T16H= tabersonine 16-hydroxylase; OMT= O-methyltransferase; NMT= N-methyltransferase; D4H= desacetoxyvindoline 4-hydroxylase; DAT = deacetylvinodline 4-O-acetyltransferase
2.3. Limitations to TIA production in *C. roseus*

The production of TIAs such as VBL and VCR is extremely low: 500 kg of dried *C. roseus* leaves are needed to produce just 1 g of VLB (Noble 1990), making their extraction expensive and these drugs cost-prohibitive (Table 2.1). Production of TIAs is low due to several reasons. First, TIAs are secondary metabolites and are only produced in a defense response, such as attack by a pathogen or wounding. Additionally, low TIA levels are largely due to spatial separation of biosynthetic sites and high degree of specialization of cells (Facchini and St-Pierre 2005). Catharanthine accumulates almost exclusively in the wax exudates on the leaf surface while vindoline is produced in specialized internal leaf cells (Roepke et al. 2010). Thus, these compounds require transport processes for their coupling to form VBL. Attempts to chemically synthesize TIAs have been unsuccessful due to their complex structure and chiral centers, and host systems such as bacteria or yeast cannot be used due to the complex and incomplete TIA biosynthetic pathway (Hughes and Shanks 2002).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Brand names</th>
<th>Manufacturers</th>
<th>Unit Price ($/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine</td>
<td>Velban ®, Velbe ®, Velsar ®, Vinblastine Sulfate</td>
<td>Bedford Laboratories, Fresenius Kabi, USA, Eli Lilly and Co., Adria Laboratories, Quad Pharmaceuticals, Hospira, Inc.</td>
<td>$16,560,000, $4,323,000, N/A, N/A, N/A, N/A</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Oncovin ®, Vincasar PFS ®, Vincristine Sulfate, Vincrex ®, Marqibo ®</td>
<td>Teva Pharmaceuticals, Hospira, Inc., Eli Lilly, Talon, USA</td>
<td>$18,060,000, $6,800,000, N/A, N/A</td>
</tr>
</tbody>
</table>

*Table 2.1: Prices of VBL and VCR based on cost per dose, from (RED BOOK Online. 2013)*
2.4. *C. roseus* cell and tissue cultures

TIA production in *C. roseus* has been studied using several forms of cultures, including callus, cell suspension, and hairy root cultures. *C. roseus* cell cultures uptake nutrients, precursors and elicitors more rapidly, therefore enhancing production of TIAs and simplifying their extraction (Mujib et al. 2012). However, TIA production is dependent on both cell differentiation and organogenesis (El-Sayed and Verpoorte 2007); specifically, some TIAs such as vindoline cannot be synthesized by *C. roseus* cell cultures since the activation of biosynthetic pathways is tissue-specific and developmentally regulated (Shukla et al. 2010). Hairy root cultures are advantageous because they grow rapidly in hormone-free media and are genetically stable differentiated cells compared to callus or cell cultures. Hairy root cultures are able to produce a broad spectrum of TIAs, and often higher levels than native roots (Bhadra et al. 1993; Jung et al. 1995). Therefore, our research employs the use of *C. roseus* hairy root cultures to study and manipulate TIA production.

2.5. Previous attempts to increase TIA production

2.5.1. Media optimization and culture conditions

Culture conditions, including medium composition, temperature, pH, and oxygenation, affect growth and production of TIAs in *C. roseus*. *C. roseus* growth media consists of inorganic and organic components, vitamins, and carbohydrates. For *C. roseus* hairy root cultures, TIA production is maximized with low levels of phosphate, nitrate, and ammonium (Schlatmann et al. 1992). Sucrose as an energy source improves hairy root growth, but does not affect TIA production (Mujib et al. 2012). While *C. roseus* growth is optimized at a higher temperature (35°C), TIA biosynthesis is highest at 25°C (Bailey and Nicholson 1990). Similarly, *C. roseus* hairy roots exhibit optimal TIA levels at an initial pH of 5.5-6.0, and under aeration conditions.
In culture vessels, different types of gases particularly CO$_2$ and ethylene are produced. The build up of CO$_2$ and ethylene in culture vessels causes a gradual loss of O$_2$ and can be detrimental for normal growth. Therefore, aeration to generates O$_2$ restores normal growth processes and regularizes alkaloid production (Lee-Parsons 2007; Mujib et al. 2012). While these conditions improve growth and TIA production, none of these strategies have increased TIA production in \textit{C. roseus} by more than 2 or 3-fold.

2.5.2. \textbf{Addition of elicitors}

An elicitor is a primary signal recognized by the plant that specifies an attack by a fungal or bacterial microorganism. Elicitors have been one of the most effective strategies to improve secondary metabolite production in plant platforms (Almagro et al. 2015). TIA production in \textit{C. roseus} is induced by both abiotic (heavy metal ions and UV light) and biotic elicitors (yeast, fungal extracts, cyclodextrins, and signaling molecules like nitric oxide and jasmonates) (Almagro et al. 2015; Mujib et al. 2012; van der Heijden et al. 2004). Among other elicitors, treatment of \textit{C. roseus} hairy root cultures with methyl jasmonate (MJ) has been vastly studied and used to significantly increase TIAS (reviewed in (Almagro et al. 2015)). For example, elicitation of \textit{C. roseus} hairy roots with MJ resulted in a 7-fold increase in ajmalicine, 2.9-fold increase in serpentine, and 3-fold increase in catharanthine (Ruiz-May et al. 2009). In our work, we therefore elicit TIA production by adding MJ to \textit{C. roseus} hairy root cultures.

2.5.3. \textbf{Metabolic engineering of TIA biosynthetic genes}

Initial genetic engineering manipulations focused on overexpressing genes coding for key enzymes in the first committed steps of the indole or terpenoid branch. In the indole pathway, for example, anthranilate synthase (AS) catalyzes the first committed step of tryptophan synthesis by converting chorismate to anthranilate. AS is a tetramer composed of two $\alpha$ subunits and two $\beta$
subunits. The α subunit catalyzes the aromatization of chorismate and is inhibited by its downstream product, tryptophan (Hughes et al. 2004a). Therefore, increased tryptophan levels inhibit native AS activity and channel metabolic flux into a competing pathway (Verpoorte et al., 1999). However, a mutation in the ASα gene yields an enzyme that is feedback-resistant to tryptophan (Hughes et al. 2004a). The overexpression of a feedback-insensitive ASα caused a 300-fold increase in tryptophan and 10-fold increase tryptamine concentrations, but only a 2-fold increase of one TIA, lochnericine (Hughes et al. 2004a). Overexpression of tryptophan decarboxylase (TDC), the first committed step in the indole pathway, led to no significant increase in tryptamine levels, but serpentine yields increased 129% (Hughes et al. 2004b). When both TDC and feedback-insensitive ASα were co-expressed, a 6-fold increase in tryptamine resulted, but no increase in downstream TIAs (Hughes et al. 2004b). Although the ASα subunit alone is able to convert anthranilate to chorismate and is responsible for tryptophan feedback inhibition, the ASβ, responsible for transferring an amino group from glutamine to the ASα subunit, also plays an important role in tryptophan synthesis (Hong et al. 2006b). In fact, expression of ASβ in conjunction with feedback-resistant ASα in C. roseus hairy roots resulted in a 4.5-fold higher resistance to tryptophan-feedback inhibition and therefore higher levels of tryptophan and tryptamine compared to the expression of ASα alone (Hong et al. 2006b). Interestingly, levels of TDC activity remained unchanged in transgenics expressing both ASα and ASβ, but tryptamine levels increased 6-fold compared to expressing ASα alone. However, ectopic expression of TDC together with mutant ASα and ASβ increased tryptamine levels further than expressing just mutant ASα and ASβ (Hong et al. 2006b). Overall, manipulations of single or multiple genes in the indole pathway increase the levels of tryptamine, but do not lead to significant increases in downstream TIAs.
Key enzymes in the terpenoid pathway have also been overexpressed. Overexpression of 1-deoxy-D-xylulose 5-phosphatesynthase (DXS) significantly increased levels of ajmalicine, serpentine and lochnericine, but significantly decreased levels of tabersonine and horhammericine (Peebles et al. 2011). When the double construct of DXS and feedback-resistant ASα were co-overexpressed, levels of horhammericine, lochnericine and tabersonine significantly increased compared to overexpressing either feedback-resistant ASα or DXS alone (Peebles et al. 2011). Similarly, when DXS was co-overexpressed with geraniol-10-hydroxylase (G10H), a limiting enzyme in the production of TIAs, a significant increase was seen in levels of ajmalicine, lochnericine and tabersonine, an overall positive gain in metabolites compared to the mixed results of overexpressing DXS alone (Peebles et al. 2011).

In addition to expressing single and multiple enzymes in the upstream pathways of TIA biosynthesis, downstream genes have also been genetically engineered in C. roseus hairy roots. For example, deacetylvindoline-4-O-acetyltransferase (DAT) catalyzes the last step of vindoline biosynthesis. DAT was overexpressed in transgenic hairy root cultures, resulting in increased levels or horhammericine (due to the interference of MAT by DAT), but levels of tabersonine and lochnericine were unaltered (Magnotta et al. 2007). These attempts to engineer key enzymes suggests evidence that engineering several genes and therefore transcription factors may be necessary for significant increases in downstream TIA production.

2.6. Regulation of TIA production by transcription factors in C. roseus

Transcription factors are proteins that bind to a specific DNA sequence and interact with the promoter of target genes to regulate the rate of initiation of mRNA synthesis by RNA polymerase II (Gantet and Memelink 2002). Despite the lack of established genetics for C. roseus, researchers have attempted to enhance TIA biosynthesis by finding transcription factors
that regulate the TIA pathway. Because transcription factors have the ability to regulate *multiple* genes, a higher level of TIA regulation can be achieved by increasing the expression of several TFs. The production of TIAs in *C. roseus* is largely due to specific transcriptional regulation of TIA biosynthetic enzymes (Memelink and Gantet 2007). Our research therefore aims to further understand the regulation of TIAs and manipulate the regulatory pathway to control and increase TIA biosynthesis.

In *C. roseus*, the presence of jasmonates activate several transcription factors, which in turn regulate the expression of key enzymes in the TIA biosynthetic pathway, leading to increased TIA production (Figure 2.4). In the absence of JA, JAZ proteins bind to the MYC2 protein and inhibit its action. However, in the presence of JA (specifically JA-Isoleucine), JAZ proteins bind to the F-box protein, COI1, to form a complex which marks JAZ proteins for rapid degradation via the 26S proteasome (Chini et al. 2007; Thines et al. 2007; Zhang 2008). MYC2 proteins then become active and are then free to induce the expression of downstream transcription factors that directly induce TIA biosynthetic genes.

The newly freed MYC2 proteins bind to the promoter region of two transcription factor genes, *Jaz* and *Orca3*. This induction of *Jaz* genes leads to *de novo* synthesis of JAZ proteins and therefore re-repression of MYC2 proteins (Chini et al. 2007; Thines et al. 2007; Zhang 2011), dampening the effect of the initial JA activation. Additionally, MYC2 activates *Orca3* gene expression by binding to the qualitative sequence or on-off switch of the *Orca3* promoter; this interaction is necessary for MJ responsiveness (Vom Endt et al. 2007; Zhang 2011). Besides the regulation of *Orca3* by MYC2, a group of AT-hook proteins bind specifically to the quantitative region of the *Orca3* promoter. These proteins are believed to determine the level of *Orca3* gene expression (Vom Endt et al. 2007).
ORCA proteins increase Str promoter activity through direct binding and activation (Menke et al. 1999a; van der Fits and Memelink 2000), and also increase the expression of several other TIA biosynthetic genes (i.e. Tdc, Cpr, Str, D4h, etc.) (van der Fits and Memelink 2000). While ORCA is an activator of TIA biosynthetic genes, the ZCT proteins are transcriptional repressors. ZCT proteins directly bind and repress the activity of Tdc and Str (Pauw et al. 2004).

Besides ORCA and ZCT, several other classes of transcription factors have been discovered which bind to TIA biosynthetic genes (specifically Tdc and Str). Two GBF transcription factors were discovered in C. roseus that bind directly to the G-box and G-box-like sequences of the Str and Tdc promoters, respectively. In vitro studies showed the binding of GBF to Str repressed its activity (Siberil et al. 2001). Another transcription factor, BPF, bound specifically to the Str promoter (and not the Tdc promoter). BPF transcription factors were not MJ responsive, however, and therefore likely regulate Str expression in a JA-independent pathway (van der Fits et al. 2000).

The WRKY1 transcription factor is believed to regulate both Orca and Zct gene expression in C. roseus. Overexpression of WRKY1 increased expression of Zct1, Zct2, and Zct3, and decreased expression of Orca2, Orca3, and Myc2 (Suttipanta et al. 2011). Besides the regulation of ZCT and ORCA, WRKY1 also binds directly to the W-box elements of the Tdc promoter, increasing its expression (Suttipanta et al. 2011).

Figure 2.4 pictures the transcription factors mentioned in this section, along with their interactions with genes and proteins they regulate. Similarly, Table 2.2 describes each transcription factor and its binding sites.
Figure 2.4. Network of transcription factors involved in JA-responsive TIA regulation. Known and potential activators are shown in green, and known/potential repressors are shown in red. Dashed lines indicate correlations that still need to be confirmed.
<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>JA responsive?</th>
<th>Binds to:</th>
<th>Specific binding site</th>
<th>Regulation:</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORCA1</td>
<td>No</td>
<td>Str</td>
<td>JERE (EMSA)</td>
<td>-</td>
<td>(Menke et al. 1999a)</td>
</tr>
<tr>
<td>ORCA2</td>
<td>Yes</td>
<td>Str</td>
<td>JERE (EMSA)</td>
<td>Str activation (trans-activation studies)</td>
<td>(Menke et al. 1999a)</td>
</tr>
<tr>
<td>ORCA3</td>
<td>Yes</td>
<td>Str</td>
<td>JERE (EMSA)</td>
<td>Str activation (trans-activation studies)</td>
<td>(van der Fits and Memelink 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tdc (EMSA)</td>
<td>-</td>
<td>Tdc activation (trans-activation studies)</td>
<td>(van der Fits and Memelink 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cpr (EMSA)</td>
<td>-</td>
<td>Cpr activation (trans-activation studies)</td>
<td>(van der Fits and Memelink 2000)</td>
</tr>
<tr>
<td>ZCT1</td>
<td>Yes</td>
<td>Tdc</td>
<td>HS and DB regions (EMSA)</td>
<td>Tdc repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Str</td>
<td>RV region containing JERE, and BA region (EMSA)</td>
<td>Str repression, specifically RV and BA repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td>ZCT2</td>
<td>Yes</td>
<td>Tdc</td>
<td>HS and DB regions (EMSA)</td>
<td>Tdc repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Str</td>
<td>RV region containing JERE, and BA region (EMSA)</td>
<td>Str repression, specifically RV and BA repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td>ZCT3</td>
<td>Yes</td>
<td>Tdc</td>
<td>All regions (EMSA)</td>
<td>Tdc repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Str</td>
<td>RV region containing JERE, and several other regions (EMSA)</td>
<td>Str repression, specifically RV and BA repression (trans-regulatory studies)</td>
<td>(Pauw et al. 2004)</td>
</tr>
<tr>
<td>MYC1</td>
<td>No</td>
<td>G-box (from Str)</td>
<td>G-box element (DNA binding specificity assay in yeast)</td>
<td>G-box activation (trans-activation studies)</td>
<td>(Chatel et al. 2003)</td>
</tr>
<tr>
<td>MYC2</td>
<td>Yes</td>
<td>Str</td>
<td>D-fragment of JRE, specifically the qualitative sequence (EMSA)</td>
<td>Orca3 activation (trans-activation, overexpression, RNAi silencing)</td>
<td>(Zhang 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G-box (yeast one-hybrid)</td>
<td>G-box activation but Str not activated (trans-activation studies)</td>
<td>(Zhang 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jaz1, Jaz2</td>
<td>Jaz1, Jaz2 expression activated (knock-down and overexpression studies)</td>
<td>(Zhang 2008)</td>
</tr>
<tr>
<td>JAZ1</td>
<td>Yes</td>
<td>MYC2 proteins</td>
<td>-</td>
<td>MYC2 activity repressed (trans-activation studies)</td>
<td>(Zhang 2008)</td>
</tr>
<tr>
<td>JAZ2</td>
<td>Yes</td>
<td>MYC2 proteins</td>
<td>-</td>
<td>MYC2 activity repressed (trans-activation studies)</td>
<td>(Zhang 2008)</td>
</tr>
<tr>
<td>Transcription Factor</td>
<td>Binding Specificity</td>
<td>Gene/Protein Bound</td>
<td>Description</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>-------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td><strong>WRKY1</strong></td>
<td>Yes</td>
<td>Tdc</td>
<td>W-box (EMSA, yeast one-hybrid) (\text{Tdc (trans-activation studies), As and Dxs expression activated; Orca2, Orca3 and Myc2 expression repressed; Zct1, Zct2 and Zct3 expression activated (overexpression and SRDX silencing studies)})</td>
<td>(Suttipanta et al. 2011)</td>
<td></td>
</tr>
<tr>
<td><strong>WRKY2</strong></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>(Suttipanta et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>AT-hook2 (2D173)</td>
<td>No</td>
<td>Orca3</td>
<td>D-fragment of JRE, specifically the quantitative sequence (EMSA)</td>
<td>(Vom Endt et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>AT-hook4 (2D38M, 2D1)</td>
<td>No</td>
<td>Orca3</td>
<td>No effect on D-fragment of JRE (trans-activation studies)</td>
<td>(Vom Endt et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>AT-hook5 (2D7)</td>
<td>No</td>
<td>-</td>
<td>D-fragment of JRE activated (trans-activation studies)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GBF1</strong></td>
<td>No (our studies)</td>
<td>Str</td>
<td>G-box element (EMSA) (\text{Str repression (trans-regulatory studies in vivo, G-box deletion analysis)})</td>
<td>(Siberil et al. 2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tdc</td>
<td>G-box-like element (EMSA) -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GBF2</strong></td>
<td>No (our studies)</td>
<td>Str</td>
<td>G-box element (EMSA) (\text{Str repression (trans-regulatory studies in vivo, G-box deletion analysis)})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tdc</td>
<td>G-box-like element (EMSA) -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BPF1</strong></td>
<td>No</td>
<td>Str</td>
<td>BA region (EMSA) -</td>
<td>(van der Fits and Memelink 2000)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2:** Summary of *C. roseus* transcription factors and the genes/proteins they bind to and regulate.
2.7. Previous attempts to manipulate transcription factors in *C. roseus* hairy roots

2.7.1. ORCA3 overexpression does not increase TIA metabolites

In *C. roseus* hairy roots, ORCA3 overexpression did not significantly increase TIA metabolites. In response to ORCA3 overexpression, the expression of *ASa*, *Dxs*, *Str*, *Sls*, and *Zcts* increased; *Tdc*, *G10h*, *Cpr*, *Gb2*, and *Orca2* remained constant; and *Sgd* decreased (Peebles et al. 2009). In another study, ORCA3 overexpression did not increase catharanthine levels, but levels of *ASa*, *Tdc*, *Str*, and *Sgd* increased (Zhou et al. 2010).

Since neither *Tdc* nor *G10h* were up-regulated when ORCA3 was overexpressed, loganin, tryptophan, or loganin and tryptophan were fed to *C. roseus* hairy roots overexpressing ORCA3. However, no significant increases in TIA metabolites were observed (Peebles et al. 2009). This may be due to the fact that *Sgd* levels did not increase with ORCA3 overexpression, and TIA metabolites measured were downstream of *Sgd*.

Surprisingly, most transcript levels of TIA pathway genes were present in lower concentrations in the ORCA3 overexpression conditions compared to the cultures elicited with JA (Peebles et al. 2009). This suggests that a negative regulator, may be overriding the positive up-regulation that normally occurs with JA elicitation for these genes; ZCTs are a potential candidate since they were upregulated by ORCA3 overexpression and can counteract the positive regulators stimulated by JA (Pauw et al. 2004; Peebles et al. 2009). Since JA caused the largest increase in the TIA transcripts and ORCA3 overexpression did not increase *G10h* and *Tdc* expression in hairy roots, overexpression ORCA3 is not an effective strategy for increasing TIA metabolite levels and the existence of other positive regulators of the TIA pathway is likely.
2.7.2. Co-overexpression of ORCA3 and G10H does not further increase catharanthine production compared to G10H overexpression alone

TIAs were not detected in ORCA3 overexpressed lines, likely because \(G10h\) expression was undetectable. G10H hydroxylates geraniol to form 10-hydroxy-geraniol, and thus controls the first committed step in the biosynthesis of secologanin and downstream TIAs. The G10H promoter contains unique binding sites of several transcriptional factors (not overlapping with the binding sites seen in \(Str\) and \(Tdc\) regulation), suggesting that the G10H promoter may be regulated by a transcription factors other than ORCA (Suttipanta et al. 2007).

\(C.\ roseus\) hairy root lines were created in which G10H or a combination of G10H and ORCA3 were overexpressed. Of the four TIAs examined, vindoline, vinblastine, and vincristine were not detectable in control or transgenic lines, but catharanthine was detected in all hairy root lines. Although catharanthine levels increased (6.5-fold) in transgenic lines expressing G10H alone and co-expressing G10H and ORCA3, there was no significant difference between the two transgenic lines despite the levels of ORCA3 being significantly higher in the G10H and ORCA3 co-overexpression lines (Wang et al. 2010a), implying that G10H plays a more important role in catharanthine synthesis. Expression of \(Str\) and \(Sls\) increased in G10H and ORCA3 overexpression lines, but expression of other genes (such as\( Asa\),\( Cpr\), and\( Tdc\)) showed no significant difference. The expression of \(D4h\) and \(Dat\) were undetectable (Wang et al. 2010a).

2.7.3. ORCA2 overexpression increased catharanthine and vindoline production

The relationship between ORCA2 and TIA biosynthesis was studied due to the fact that ORCA2 binds and activates \(Str\) expression (Menke et al. 1999a). When ORCA2 was overexpressed in \(C.\ roseus\) hairy root cultures, levels of tryptamine, ajmalicine, serpentine, catharanthine, 16-hydroxytabersonine and 19-hydroxytabersonine increased while levels of tabersonine,
strictosidine and horhammericine decrease (Li et al. 2013). These metabolite increases were associated with an increase in $\text{A5a, Tdc, G10h, Str, T16h,}$ and $D4h$ expression. Expression levels of $\text{Dxs, Cpr,}$ and $16omt$ were unaffected, and levels of $\text{Sgd}$ and $\text{Dat}$ decreased (Li et al. 2013). Interestingly, levels of $\text{Orca3, Zct1, Zct2,}$ and $\text{Zct3}$ increased as well (Li et al. 2013). The increase in $\text{Zct}$ expression is consistent with overexpression of the homologous transcription factor, ORCA3 (Peebles et al. 2009).

2.7.4. **WRKY1 overexpression increased levels of serpentine**

WRKY transcription factors, containing a WRKY amino acid domain and DNA-binding sequence known as the W-box (TTGACC/T), form a large, plant-specific family of proteins (Rushton 2010). When WRKY1 was overexpressed in $\text{C. roseus}$ hairy roots, levels of serpentine significantly increased, but levels of catharanthine significantly decreased (Suttipanta et al. 2011). These changes were correlated with significantly increased $\text{Tdc}$ levels and increased (to a lesser extent) levels of $\text{As, Dxs, Sls}$ and $\text{Sgd}$ (Suttipanta et al. 2011). Interestingly, levels of $\text{Zct1, Zct2}$ and $\text{Zct3}$ were also increased, but levels of $\text{Orca2, Orca3}$ and $\text{Myc2}$ were significantly decreased (Suttipanta et al. 2011). These trends were confirmed by repressing WRKY1 (and homologous protein) activity through fusing the SRDX repression domain to WRKY1 (Suttipanta et al. 2011).

In transgenic lines overexpressing WRKY2, levels of serpentine, tabersonine, and catharanthine increased (Suttipanta 2011). WRKY2 overexpression increased levels of $\text{Tdc, Str, Sgd, D4h, Dat,}$ and $\text{Mat}$, but transcript levels of $\text{As, Dxs, Cpr}$ and $\text{Prx}$ decreased (Suttipanta 2011). Up-regulation of transcription factors was also observed in these lines: $\text{Orca2, Orca3, Zct1, Zct3,}$ and $\text{Wrky1}$ expression increased. Levels of $\text{Zct2, Ghf1}$ and $\text{Ghf2}$ only slightly decreased (Suttipanta 2011).
2.8. Our strategy for increasing TIA production

Since MJ had a more significant increase on TIA production than overexpressing ORCA, our research focuses on the combined strategy of eliciting transcriptional activators through elicitation with MJ while simultaneously silencing the ZCT transcriptional repressors in hairy roots. We anticipate this novel, combinatorial approach will amplify multiple enzymes in the biosynthetic pathway, and therefore significantly increase TIA production in *C. roseus* hairy roots. Importantly, this research will elucidate more about the transcription factor network in *C. roseus* and contribute to fundamental understanding of the regulation of TIA biosynthesis. This knowledge will provide the basis for genetic engineering and new strategies for increasing production in hairy root cultures that can then be applied to other plant systems with important products.
3.0 Experimental Methods

3.1. *C. roseus* hairy root media preparation

Liquid media was prepared and filter-sterilized one week prior to subculturing *C. roseus* hairy roots. The liquid media consisted of sucrose (30 g/L; Sigma-Aldrich Corp.), half-strength Gamborg’s B-5 Basal Salt mixture (1.55 g/L; #G5768, Sigma-Aldrich), and full strength Gamborg’s vitamin solution (1 mL/L; #G1019, Sigma-Aldrich). The pH was adjusted to 5.7 with 1N sodium hydroxide and the media was filter-sterilized using a prefilter (Millipore Millex Nonsterile Syringe Prefilters; #SLAP05010, Fisher Scientific) and a 0.22µm filter unit (Millipore Sterivex Sterile Pressure-Driven Devices; #SVGV010RS, Fisher Scientific) into sterile 1L media bottles. Alternatively, the media was autoclaved (30 min, 121°C) prior to the addition of Gamborg’s vitamins. The media was stored at 4°C until use.

*C. roseus* hairy roots were also maintained on plates. The solid media for the plates was prepared similarly (30 g/L sucrose, 1.55 Gamborg’s B-5 Basal Salt mixture, pH adjusted to 5.7), with agar (6 g/L; part # A-4675, Sigma-Aldrich). The media was autoclaved at 121°C for 30 min. Once the autoclaved media was cool to touch, Gamborg’s vitamin stock solution (1 mL/L; #G1019, Sigma-Aldrich) was filter-sterilized (Corning; #431224, Fisher Scientific) and added to the media. The solid media was poured into sterile plates (Corning; #430591, Fisher Scientific). The plates were wrapped with parafilm and stored at 4°C until use.

3.2. *C. roseus* hairy root culture subculture

Liquid media (50mL) was distributed into sterile 250mL Erlenmeyer flasks. Root tips from existing hairy root cultures were cut into 3-4cm long pieces. Five white, young, actively-growing root tips were placed into each flasks containing 50mL of media and capped with Bellco foam.
closures. The cultures were kept in a dark incubator-shaker (Forma Scientific; Model # 4584; Marietta, OH) maintained at 26°C and 100 rpm. Sterile water was added each week to compensate for evaporation loss. This subculture was performed every 28 days.

*C. roseus* hairy roots were also maintained on solid media as a back up. Five 3-4 cm long, white and actively growing tips were also placed on plates containing media. The plates were maintained in the dark at room temperature.

### 3.3. Characterization of *C. roseus* hairy root growth

To characterize the growth, *C. roseus* hairy root cultures were monitored over a 40-day period. *C. roseus* hairy roots were subcultured into 18 flasks and maintained as described previously. Hairy root cultures were removed from the media, blotted dry, and the fresh weight was recorded every 5 days for 40 days of growth. Hairy root samples were frozen at -20°C and then lyophilized using a Flexi-Dry MP Freeze Dryer (Kinetics Thermal Systems; FD-3-85-A-MP) for 48 h. The dry weight of each culture was recorded.

Exponential growth occurs between days 15-25 of the subculture period (Figure 3.5 and Figure 3.6). Therefore, early-exponential growth phase (day 21) was used for elicitation experiments, and the end of the exponential growth phase (day 28) was used as the subculture period.
Figure 3.5: *C. roseus* hairy root growth over 30 days

Figure 3.6: *C. roseus* hairy root growth curve, including fresh and dry weight concentrations. Error bars represent standard deviations of two biological replicates.
3.4. Elicitation of hairy root cultures with methyl jasmonate (MJ)

Stock solutions of MJ (assay > 95%; #W341002; Sigma-Aldrich) were prepared in ethanol (200 proof) and filter-sterilized using sterile syringe filters (#431224; Fisher-Scientific). On day 21 (or day 26 for slower-growing transgenics) of the C. roseus growth cycle (early-exponential phase), the sterile MJ was added to each hairy root culture flask, assuming the total volume was 50mL in each flask, to reach the desired final concentrations. Calculations were done using the MJ molecular weight = 224.3 g/mol and MJ density = 1.03 g/mL. Since MJ is volatile, elicited hairy root cultures were stored in a different incubator maintained at the same conditions (dark, 26°C and 100 rpm).

3.5. Extraction of TIA s from C. roseus hairy roots

C. roseus hairy roots were removed from flasks, blotted dry, and frozen at -20°C. Frozen hairy root samples were lyophilized using a Flexi-Dry MP Freeze Dryer (Kinetics Thermal Systems; FD-3-85-A-MP) for 48 h and the dry weight was recorded. Using a mortar and pestle, the dried hairy root cultures were ground into a fine, white powder. 50mg of dry powder was extracted with 5mL of HPLC grade methanol in 15mL centrifuge tubes by first being vortexed, then sonicated (Sonicator FS14H; Fisher-Scientific) for 30 min. The samples were vortexed again, then centrifuged for 30 min at 5,000 x g at 25°C. The supernatant, containing the TIA s, was then transferred to a new 15mL tube and concentrated using a Savant SpeedVac Plus Concentrator (Thermoquest; #SC210A; Holbrook, NY). The remaining tissue sample was re-extracted with another 5mL of methanol, and the resulting supernatant was combined with the first extract; the samples were concentrated overnight. The dried TIA extracts were then re-dissolved in 1mL of HPLC grade methanol and filter-sterilized using non-sterile syringe filters (Millipore Millex
Nonsterile Syringe Filters; #SLHN013NL) into HPLC vials (Waters; #186000384C, 12 x 32 mm glass screw).

3.6. TIA\textsubscript{s} analysis by HPLC

To determine the TIA concentrations in the \textit{C. roseus} hairy root culture, extracted samples were analyzed through a High Performance Liquid Chromotography (HPLC) system (Waters 2695 Separations Module, Waters 996 Photodiode Array Detector, Millennium 32 Software; Waters Corp., Millford, MA) in which compounds were separated using a reversed-phase C18 column (Luna, 150 x 4.60 mm ID column, 5 mm particle size, Phenomenex, Torrance, CA).

The HPLC mobile phases for TIA separation were: 99.9\% water with 0.1\% (v/v) formic acid as the aqueous phase, and 99.9\% acetonitrile with 0.1\% (v/v) formic acid as the organic phase. The protocol consisted of the following steps: 1) 90\% aqueous and 10\% organic as the initial condition, 2) gradient to 70\% aqueous and 30\% organic over 20 min, 3) gradient to 100\% organic over 8 min, 4) gradient to 90\% aqueous and 10\% organic over 10 min, and 5) isocratically at 90\% aqueous and 10\% organic for 20 min to equilibrate the column for the next injection. All the flow rates were maintained at 1.0 mL/min.

Ajmalicine (254nm), serpentine (254nm), strictosidine (274nm), tabersonine (329nm), and tabersonine-like compounds (329nm) were monitored at their respective wavelengths. These TIA\textsubscript{s} were quantified by comparing peaks to their respective standard curves (Figure 3.7), which were generated with each HPLC run, and UV spectra (Figure 3.8). These standard curves (or approximated Beer’s Law fitting for unattainable standards) were used to calculate TIA concentrations in \textit{C. roseus} hairy root samples by relating metabolite peak area to concentration.
Figure 3.7: HPLC calibration curves of TIAs strictosidine (at 274nm), ajmalicine (at 254nm), tabersonine (at 329nm) and strictosidine (at 274nm)

Figure 3.8: UV spectra for strictosidine (274nm, 12.988 min), ajmalicine (254nm, 14.925 min), tabersonine (329nm, 15.891 min), and serpentine (254nm, 15.935 min).
3.7. Validation of TIA extraction and HPLC methods

To test the reproducibility of the HPLC, three injections of a tabersonine standard were run with 10µL and 2.5µL injection volumes. There is less than 2% standard deviation in the peak area between each of the three injections, indicating strong reproducibility of the HPLC protocol (Table 3.3). Also, using a larger injection volume (10µL instead of 2.5µL) results in better reproducibility. To similarly test the reproducibility of the HPLC with a complex mixture, two biological replicates were elicited with 250µM MJ on day 21 of the growth cycle and were harvested five days after elicitation. Three injections of each biological replicate were run on the HPLC. There is less than 2% standard deviation between each injection, which indicates the reproducibility of the HPLC with a complex mixture (Table 3.4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Injection</th>
<th>Injection volume (µL)</th>
<th>Retention time (min)</th>
<th>Peak area</th>
<th>%StDev of 3 injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2mg/mL tabersonine standard</td>
<td>1</td>
<td>10</td>
<td>29.0</td>
<td>4961885</td>
<td>0.37%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>29.6</td>
<td>4968848</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>29.8</td>
<td>4934290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.5</td>
<td>30.4</td>
<td>1049745</td>
<td>1.74%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.5</td>
<td>30.6</td>
<td>1085443</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.5</td>
<td>30.6</td>
<td>1058733</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: HPLC reproducibility of multiple tabersonine standard injections and injection volumes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Injection</th>
<th>Ajmalicine concentration (mg/L culture)</th>
<th>%StDev of 3 injections</th>
<th>Tabersonine concentration (mg/L culture)</th>
<th>%StDev of 3 injections</th>
<th>Strictosidine concentration (mg/L culture)</th>
<th>%StDev of 3 injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>250uM MeJA biological replicate 1</td>
<td>1</td>
<td>4.90</td>
<td>1.89%</td>
<td>17.81</td>
<td>0.40%</td>
<td>18.16</td>
<td>1.20%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.90</td>
<td></td>
<td>17.68</td>
<td></td>
<td>18.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.06</td>
<td></td>
<td>17.70</td>
<td></td>
<td>18.45</td>
<td></td>
</tr>
<tr>
<td>250uM MeJA biological replicate 2</td>
<td>1</td>
<td>3.53</td>
<td>0.98%</td>
<td>9.43</td>
<td>0.97%</td>
<td>14.89</td>
<td>0.45%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.59</td>
<td></td>
<td>9.25</td>
<td></td>
<td>14.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.53</td>
<td></td>
<td>9.30</td>
<td></td>
<td>14.76</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: HPLC reproducibility of multiple C. roseus hairy root samples for multiple injections
The reproducibility of the TIA extraction from *C. roseus* hairy root cultures was analyzed by extracting each biological replicate three times (50mg each) and running each extract on the HPLC. There was 15 - 24% variability in concentration between the extractions of the same biological replicate (Table 3.5). When comparing the reproducibility between the two biological replicates, there was 15 – 44% standard deviation in concentration between the two biological replicates (Table 3.6).

### Table 3.5: Reproducibility of various extractions of *C. roseus* hairy root samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction</th>
<th>Ajmalicine concentration (mg/L culture)</th>
<th>%StDev of 3 extractions</th>
<th>Tabersonine concentration (mg/L culture)</th>
<th>%StDev of 3 extractions</th>
<th>Strictosidine concentration (mg/L culture)</th>
<th>%StDev of 3 extractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>250uM MeJA biological replicate 1</td>
<td>1</td>
<td>3.30</td>
<td>15.16%</td>
<td>17.94</td>
<td>20.16%</td>
<td>19.5592</td>
<td>23.66%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.80</td>
<td></td>
<td>13.63</td>
<td></td>
<td>15.4218</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.44</td>
<td></td>
<td>12.30</td>
<td></td>
<td>12.1420</td>
<td></td>
</tr>
<tr>
<td>250uM MeJA biological replicate 2</td>
<td>1</td>
<td>3.02</td>
<td>16.82%</td>
<td>9.05</td>
<td>19.33%</td>
<td>15.0591</td>
<td>22.28%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.34</td>
<td></td>
<td>6.61</td>
<td></td>
<td>10.5367</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.23</td>
<td></td>
<td>6.54</td>
<td></td>
<td>10.3405</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.6: Reproducibility between biological replicates of *C. roseus* hairy root samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ajmalicine concentration (mg/L culture)</th>
<th>%StDev of 2 biological replicates</th>
<th>Tabersonine concentration (mg/L culture)</th>
<th>%StDev of 2 biological replicates</th>
<th>Strictosidine concentration (mg/L culture)</th>
<th>%StDev of 2 biological replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>250uM MeJA biological replicate 1</td>
<td>4.96</td>
<td>23.31%</td>
<td>17.73</td>
<td>43.94%</td>
<td>18.40</td>
<td>15.28%</td>
</tr>
<tr>
<td>250uM MeJA biological replicate 2</td>
<td>3.55</td>
<td></td>
<td>9.33</td>
<td></td>
<td>14.82</td>
<td></td>
</tr>
</tbody>
</table>

### 3.8. RNA extraction from *C. roseus* hairy roots

RNA was extracted from *C. roseus* hairy root cultures using the RNAzol®RT (#RN 190; Molecular Research Center, Inc.) method. RNAzol®RT is an effective reagent for isolation of total RNA and mRNA from samples of human, animal, plant, bacterial and viral origin. It allows for isolation of pure and un-degraded RNA.
To isolate the mRNA fraction, first the tissue was homogenized in a mortar and pestle. The homogenized tissue (~100mg) was added to a 1.5mL eppendorf tube, along with 800µL of RNAzol®RT buffer and mixed by inverting. To precipitate the DNA, proteins and polysaccharides, 0.4mL of nuclease-free water was added to the lysate. The samples were then vortexed for 15 seconds each and stored at room temperature for 15 min. Next, the samples were centrifuged at 12,000 x g for 15 min. Following centrifugation, DNA, proteins and most polysaccharides formed a semisolid pellet at the bottom of the tube, while the RNA remained soluble in the supernatant.

The supernatant (~800µL) was transferred to a new tube, leaving a layer of the supernatant above the DNA/protein pellet. The RNA was then precipitated by mixing 0.4mL of 75% ethanol (v/v) with the transferred 800µL of supernatant. This was stored for 10 min and then centrifuged at 12,000 x g for 10 min. mRNA precipitate forms a white pellet at the bottom of a tube. The supernatant was carefully removed from each tube without disturbing the mRNA pellet at the bottom. The mRNA pellet was then washed twice by mixing it with 500µL of 75% ethanol (v/v), and centrifuging 8,000 x g for 5 min. The ethanol was removed using a micropipette, and the mRNA pellet was finally dissolved in 20-30µL of nuclease-free water.

The extracted mRNA was quantified using NanoDrop (ND-1000 Spectrophotometer). The absorbance ratio A$_{260}$/A$_{280}$ was also measured to assess the purity of each mRNA sample. Because DNA and RNA absorb at 260 nm and proteins absorb at 280 nm, the desired A$_{260}$/A$_{280}$ ratio should be between 1.8 and 2.1. Ratios below 1.8 indicate possible protein contamination. Ratios above 2.1 indicate the presence of degraded RNA or excess free nucleotides.
3.9. First strand cDNA synthesis from mRNA

Complementary DNA (cDNA) is synthesized *in vitro* from a mRNA template using the reverse transcriptase enzyme by a process called reverse transcription (RT). This is done in order to analyze the expression of genes through PCR or qPCR. mRNA extracted from *C. roseus* hairy roots was converted to cDNA using an Invitrogen kit (SuperScript First-Strand Synthesis System for RT-PCR; #11904-018). Since plant mRNA contain poly(A) tails, an oligo(dT) primer is used to synthesize cDNA. The RNA samples (0.2-5µg) were combined with dNTP mix (1µL) and oligo(dT) primer (1µL); the final volume in each of the tubes was brought to 10µL with nuclease-free water. The samples were then incubated at 65°C for 5 min to denature the RNA and placed on ice for at least 1 min. For each RNA sample, a reaction mixture containing 10XRT buffer (2µL), 25mM MgCl₂ (4µL), 0.1M dithiothreitol (DTT, 2µL), and RNaseOUT Recombinant RNase inhibitor (1µL) was prepared. The RNaseOUT™ inhibitor was added to prevent RNase from digesting the RNA since RNase is active at 37 ± 7°C. The reaction mixture (9µL) was added to each tube, vortexed quickly and centrifuged. These tubes were then incubated at 42°C for 2 min for the primer to anneal. The SuperScript II™ RT enzyme (1µL) was then added to each reaction (except the “no-RT” controls); the tubes were vortexed quickly, centrifuged down, and incubated at 42°C for 50 min to complete the cDNA synthesis.

The reaction was terminated by incubating the samples at 70°C for 15 min. The samples were then chilled on ice for 1-5 min. RNase H (1µL), which degrades the original mRNA template to yield single-stranded cDNA, was added to each reaction and the samples were incubated at 37°C for 20 min. This completed the cDNA synthesis, and the cDNA samples were stored at -80°C for future use for qPCR.
3.10. Designing and verifying qPCR primers

Primers are short, single-strands of nucleic acids that serve as the starting point for DNA replication during quantitative real-time polymerase chain reaction (qPCR) since the enzymes that catalyze replication, DNA polymerases, can only add new nucleotides to an existing strand of DNA. Because the quantification in qPCR depends on the formation of double-stranded DNA, the chosen primers must be specific to the gene monitored so that the correct product is obtained and monitored, and the primer pair (forward and reverse) must amplify the gene product in a non-biased manner.

Primers were therefore designed for the genes encoding *C. roseus* biosynthetic genes and transcription factors. The National Center for Biotechnology Information database (NCBI Pubmed, www.pubmed.gov) was used to identify the coding DNA sequence (CDS) of each gene sequence, and Integrated DNA Technologies (IDT; www.idtdna.com) was used to design a specific primer pair for each gene. The following parameters were used in designing primers on IDT: 24 base-pairs for primer size, 60°C for primer melting temperature, 50% for GC content, and 150-250 base-pairs as the product size. The NCBI Basic Local Alignment Search Tool (BLAST) was used to verify that the primer pair was unique to this gene of interest. Primers were ordered from IDT.

Primers were first verified by checking for the correct size of the qPCR product as well as a single product. First, DNA Loading Buffer (3µL) was added to the qPCR products (10µL), and this was then loaded in a 1-1.5% agarose gel, alongside a 100 bp DNA ladder (5µL; #N32315). The gel was run at 100 volts for 60 min and viewed using a UV transilluminator.

If the qPCR products formed a single product of the correct size, the product was then extracted from the agarose gel using the Zymoclean™ Gel DNA Recovery Kit (#D4001; Zymo
Research Corp.). Using a razor blade, the DNA fragment was excised from the agarose gel, weighed, and put into a 1.5mL microcentrifuge tube. Three volumes of the Agarose Dissolving Buffer (ADB) was added to the sample, and the sample was incubated at 55°C for 10 min, with intermittent vortexing, until the gel slice was completely dissolved. The melted agarose solution was transferred to a Zymo-Spin™ Column in a Collection Tube and centrifuge for 60 sec at 16,000 x g, following which the flow-through was discarded. Then 200µL of DNA Wash Buffer was added to the column, centrifuged for 30 sec at 16,000 x g, and the flow-through was discarded. This wash step was repeated twice. DNA Elution Buffer (10µL) was added directly to the column matrix, the column was then placed into a new 1.5mL microcentrifuge tube and centrifuged for 60 seconds at 16,000 x g to elute the DNA. The purified DNA was then quantified using NanoDrop (ND-1000 Spectrophotometer) by measuring the DNA concentration in ng/µL. The purified products were then sent to GENEWIZ (Cambridge, MA) for sequencing, and the formation of the correct primer product for each primer pair was confirmed.

To ensure accurate quantification in qPCR, the designed primers should achieve 100% amplification efficiency. As the qPCR primers deviate from this ideal, the error in the fold difference increases exponentially. Therefore, the traditional method is to determine the amplification efficiency using a calibration curve. The calculations for efficiency are based on the fundamental qPCR concept that each strand of template DNA yields two double-stranded DNA strands. Therefore, at the end of n cycles:

Number of DNA copies = $2^n$ copies

If $X_0$ = the initial number of DNA molecules, the above equation can be written as:

$X_n = X_0(2)^n =$ number of DNA copies after cycle n
If the efficiency of amplification is denoted by $E$, particularly in the case that one strand of template DNA does not yield two DNA copies and the efficiency is less than 100%, the above equation can be written as:

$$X_n = X_0(1+E)^n$$

If change in cycle number is denoted by $\Delta Ct$, such that,

$$\Delta Ct = \text{Ct of DNA sample after cycle } n - \text{Ct of DNA sample initially}$$

Then,

$$X_n = X_0(1+E)^{\Delta Ct}$$

Thus,

$$X_0 = X_n(1+E)^{-\Delta Ct}$$

The linear form of this equation is:

$$\Delta Ct = \frac{-1}{\log(1 + E)} \log(X_0) + \frac{\log(X_n)}{\log(1 + E)}$$

Therefore, plotting $\Delta Ct$ vs. $\log(X_0)$, will yield a slope of $\frac{-1}{\log(1 + E)}$, from which the efficiency ($E$) can be calculated.

To create this calibration curve, a series of dilutions of cDNA (diluted 1:4, 1:16, 1:64, and 1:256) were run in a qPCR assay using primers specific for each gene. The $\Delta Ct$ values (relative to the lowest dilution) were plotted against the initial dilution ($X_0$) of input material on a semi-log$_{10}$ plot. The slope of the series was calculated and used to determine the efficiency of the amplification. For example, the primers for the Orca3 gene yielded a slope of -3.3413 (Figure 3.9: Sample calculation and efficiency plot for designed Orca3 primers), therefore $E = 99.2\%$. Similarly, an efficiency of ~100\% (+/- 5\%) was achieved with the primer set for each gene monitored, or the primers were redesigned.
### Table 3.9: Sample calculation and efficiency plot for designed Orca3 primers

<table>
<thead>
<tr>
<th>Sample dilution</th>
<th>Concentration ratio ($X_0$)</th>
<th>$\log (X_0)$</th>
<th>Average Ct</th>
<th>StDev Ct</th>
<th>$\Delta$Ct (relative to Ct for 1/4 dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4</td>
<td>0.25000</td>
<td>-0.60206</td>
<td>21.157</td>
<td>0.252</td>
<td>0.00</td>
</tr>
<tr>
<td>1/16</td>
<td>0.06250</td>
<td>-1.20412</td>
<td>23.333</td>
<td>0.127</td>
<td>2.18</td>
</tr>
<tr>
<td>1/64</td>
<td>0.01563</td>
<td>-1.80618</td>
<td>25.020</td>
<td>0.327</td>
<td>3.86</td>
</tr>
<tr>
<td>1/256</td>
<td>0.00391</td>
<td>-2.40824</td>
<td>27.300</td>
<td>0.291</td>
<td>6.14</td>
</tr>
</tbody>
</table>

![Orca3 efficiency curve](image1.png)

$y = -3.3413x - 1.9833$

$R^2 = 0.99698$

**Figure 3.9:** Sample calculation and efficiency plot for designed Orca3 primers

### 3.11. Monitoring gene expression using qRT-PCR (qPCR)

Quantitative real time reverse-transcriptase PCR (qPCR) was used to quantify changes in specific gene expression levels in *C. roseus* hairy root cultures. qPCR reactions were prepared using RT² SYBR® Green/ROX qPCR Mastermix (#330522; SABiosciences), which includes *Taq* DNA polymerase, dNTPs, MgCl₂, and SYBR Green dye. The SYBR Green dye fluoresces when bound to double-stranded DNA, and is therefore used as the means of quantification. Reaction mixtures for the qPCR were prepared in MicroAmp® Fast 8-Tube Strip (0.1 mL, #4358293; Life Technologies) and optical strip caps (#4323032; Life Technologies). Each
reaction consisted of: SYBR Green Master Mix (12.5µL), forward primer, downstream primer, sample cDNA (1µL), and nuclease-free water (to a total volume of 25µL). The samples were mixed and centrifuged. qPCR was performed using QuantStudio™ 6 Flex Real-Time PCR System (#4485691; Life Technologies). The initial thermocycler protocol set-up consisted of a heating cycle at 95°C for 10 min. The thermocycler was then set for 40 cycles; each cycle consisted of 1) 95°C for 30 sec for denaturation, 2) 60°C for 45 sec for the primers to anneal, and 3) 72°C for 60 sec for primer extension, after which the detection took place. After 40 cycles, the extension step at 72°C was repeated for another 3 min followed by a final cooling step to 4°C.

The amplification plot depicts the amount of product formed with respect to the number of cycles (Ct value). Since the SYBR Green dye only binds to double-stranded DNA, the fluorescence increases exponentially with increased product formation in each cycle, and reaches a plateau when one of the reactants becomes limiting. A dissociation plot is also generated when the qPCR products are subjected to an increase in temperature; this plot indicates any non-specific products formed, as well as primer-dimers. The controls (no-template control does not contain any cDNA, and no-reverse-transcriptase control does not contain any SuperScript enzyme) should not result in any amplification or dissociation plots. The no-template control checks for any DNA contamination in the reagents, while the no-reverse-transcriptase (no-RT) control checks for genomic DNA contamination.

3.12. qPCR data analysis using the \( \Delta \Delta \text{Ct} \) method

The \( \Delta \Delta \text{Ct} \) algorithm is a convenient method to analyze the relative changes in gene expression. It requires at least one housekeeping gene, which is assumed to be uniformly and constantly expressed in all samples under all treatments, as well as one reference sample (usually an untreated sample). The expression of other samples is then compared to the reference sample.
The exponential amplification of the qPCR can be described by the equation:

\[ X_n = X_0 (1+E)^n \]  

(1)

Where \( X_0 \) = the initial number of DNA molecules,

\( X_n \) = number of DNA copies after cycle \( n \)

\( E \) = amplification efficiency

If \( n \) represents the Ct of the cycle at which the target gene reaches a particular threshold, the above equation (1) can be written as:

\[ X_{Ct} = X_0 (1+E)^{Ct} = K_{Ct} \]  

(2)

Where \( K_{Ct} \) is a constant.

Since the expression level for the gene of interest is normalized relative to \( Rps9 \) (the expression level of \( Rps9 \) does not change with respect to the environmental conditions or treatment conditions, section 3.13), an equation similar to equation (2) can also be written for \( Rps9 \):

\[ R_{Ct,R} = R_0 (1+E)^{Ct,R} = K_{Ct,R} \]  

(3)

Where \( R_{Ct,R} \) = number of DNA copies for \( Rps9 \) after Ct cycles

\( R_0 \) = number of DNA copies for \( Rps9 \) present initially

\( Ct,R \) = number of PCR cycles for \( Rps9 \)

Dividing equation (2) by (3) yields:

\[ \frac{X_{Ct}}{R_{Ct,R}} = \frac{X_0 (1+E)^{Ct}}{R_0 (1+E)^{Ct,R}} = \frac{K_{Ct}}{K_{Ct,R}} = K \]

Assuming the amplification efficiency is same for the target gene and housekeeping (\( Rps9 \)) gene,

\[ \frac{X_0 (1+E)^{Ct-Ct,R}}{R_0} = \frac{X_0 (1+E)^{\Delta Ct}}{R_0} = K \]
If $X = \frac{X_0}{R_0}$, and $\Delta C_t = C_t - C_t$, R, then:

$$X = K(1 + E)^{\Delta C_t} \quad (4)$$

Where $C_t = C_t$ for the target gene

$C_t, R = C_t$ for Rps9

When comparing the expression levels of transcription factor and TIA biosynthetic genes in MJ-treated cultures to the expression level for the untreated cultures, equation (4) can be written for the treated and untreated cultures:

$$X_{\text{Treated}} = K(1 + E)^{\Delta C_{t\text{Treated}}} \quad (5)$$

$$X_{\text{Untreated}} = K(1 + E)^{\Delta C_{t\text{Untreated}}} \quad (6)$$

Dividing equation (5) by (6):

$$\frac{X_{\text{Treated}}}{X_{\text{Untreated}}} = \frac{(1 + E)^{\Delta C_{t\text{Untreated}}}}{(1 + E)^{\Delta C_{t\text{Treated}}}} = (1 + E)^{\Delta\Delta C_t}$$

Where $\Delta\Delta C_t = \Delta C_{t\text{Untreated}} - \Delta C_{t\text{Treated}}$

Therefore, the amount of target gene (transcription factor or TIA biosynthetic gene, in our case), normalized to the housekeeping gene and relative to an untreated reference sample, is given by:

$$\frac{X_{\text{Treated}}}{X_{\text{Untreated}}} = 2^{\Delta\Delta C_t}$$

Assuming the efficiency of both the target gene and housekeeping gene ($Rps9$) is 100% (see section 3.10).
3.13. *Rps9* as a housekeeping gene for *C. roseus*

*Rps9*, which codes for the 40S ribosomal protein S9, was used as the housekeeping gene for all qPCR analysis. In order to verify that *Rps9* accurately serves as a housekeeping gene, the expression of *Rps9* was monitored in *C. roseus* hairy root cultures elicited with both 250µM and 1000µM MJ over an 8-hour timecourse. Compared to the untreated (0 h) control, the expression of *Rps9* (represented by Ct value) remain relatively constant and are not affected by MJ-treatment or dosage over an 8-hour time course. (Figure 3.10). The slight variations in Ct values are likely due to small differences in the amount extracted and standard error of the NanoDrop (ND-1000 Spectrophotometer) quantification. Because *Rps9* does not change with respect to the environmental or treatment conditions, it was used as a housekeeping gene to normalize gene expression using the ΔΔCt analysis.

![Graph showing the expression of Rps9](image)

**Figure 3.10**: Expression of Rps9 is not affected by MJ-treatment or dosage over time
3.14. Validation of RNA extraction and cDNA synthesis

To measure reproducibility of the RNA extraction and cDNA synthesis methods, three biological replicates of *C. roseus* hairy root cultures were elicited with 1000µM MJ on day 21 of the growth cycle and were harvested after 24 h, along with three untreated samples. RNA was extracted from all samples, and cDNA was synthesized. To first test the reproducibility of the RNA extraction, two aliquots were extracted each from one untreated biological replicate and one biological replicate treated with 1000µM MJ. The expression of three transcription factor genes, *Jaz1*, *Orca3* and *Zct1*, was monitored in each extract. The RNA extraction reproducibility ranges between 2-20% and varies between the genes monitored (Table 3.7). To test the reproducibility of gene expression between biological replicates, 3 biological replicates each of untreated and 1000µM MJ-treated samples was analyzed. There was 10-35% standard deviation in fold increase between the three biological replicates of both treatments for the same genes monitored (Table 3.8).

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA Extraction</th>
<th><em>Jaz2</em> Fold Increase</th>
<th>% StDev of Fold Increase of 2 extractions</th>
<th><em>Orca3</em> Fold Increase</th>
<th>% StDev of Fold Increase of 2 extractions</th>
<th><em>Zct1</em> Fold Increase</th>
<th>% StDev of Fold Increase of 2 extractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated, biological replicate 1</td>
<td>1</td>
<td>1.0</td>
<td>9.2%</td>
<td>1.0</td>
<td>13.7%</td>
<td>1.0</td>
<td>11.5%</td>
</tr>
<tr>
<td>Untreated, biological replicate 2</td>
<td>2</td>
<td>0.9</td>
<td>1.2</td>
<td>0.9</td>
<td>1.2</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Untreated, biological replicate 3</td>
<td>1</td>
<td>129.6</td>
<td>20.5%</td>
<td>26.4</td>
<td>7.9%</td>
<td>14.7</td>
<td>1.9</td>
</tr>
<tr>
<td>1000µM MJ, biological replicate 1</td>
<td>2</td>
<td>96.8</td>
<td></td>
<td>29.6</td>
<td></td>
<td>14.3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.7: Reproducibility of RNA extraction**

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>Jaz2</em> Fold Increase</th>
<th>% StDev of Fold Increase of 3 biological replicates</th>
<th><em>Orca3</em> Fold Increase</th>
<th>% StDev of Fold Increase of 3 biological replicates</th>
<th><em>Zct1</em> Fold Increase</th>
<th>% StDev of Fold Increase of 3 biological replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated, biological replicate 1</td>
<td>1.0</td>
<td>32.3%</td>
<td>1.0</td>
<td>34.9%</td>
<td>1.0</td>
<td>30.3%</td>
</tr>
<tr>
<td>Untreated, biological replicate 2</td>
<td>1.3</td>
<td>0.5</td>
<td>0.5</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated, biological replicate 3</td>
<td>1.9</td>
<td>1.1</td>
<td>1.1</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000µM MJ, biological replicate 1</td>
<td>129.6</td>
<td>35.5%</td>
<td>26.4</td>
<td>10.0%</td>
<td>14.7</td>
<td>35.3%</td>
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<tr>
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<td>63.5</td>
<td>21.7</td>
<td>21.7</td>
<td>29.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.8: Reproducibility of gene expression between biological replicates**
3.15. Development of stable transgenic *C. roseus* hairy root cultures

3.15.1. Growth of *C. roseus* seedlings

The seedling growth media consisted of Gamborg’s B-5 salts (3.1 g/L, Caisson Labs), Gamborg’s 1000X vitamins (1 mL/L, Sigma-Aldrich), pH adjusted to 5.7, and agar (6 g/L, Sigma-Aldrich). Media was autoclaved for 30 min, and filter-sterilized vitamins were added after the autoclaved media was cool. The media was poured ~1 inch thick into sterile Magenta GA-7 boxes (Magenta Corporation).

*C. roseus* seeds (Vinca Little Bright Eye, NEseed) were surface-sterilized by using 80% (v/v) ethanol (200 proof, Decon Labs Inc.) for 30 s, followed by 30% (v/v) bleach (Chlorox Company) containing 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 5 min. The seeds were then rinsed with sterile water five times and soaked in sterile water for 24 h prior to being planted. Seedlings were planted ~2 mm below the agar surface of the prepared GA-7 vessels and grown in the dark for one week and then under a 16 h light/8 h dark cycle (24 Watt Daylight, 6400 K, Pegasus Associates Lighting).

3.15.2. Preparation of recombinant plasmids

All cloning/electroporation methods were performed by Jessica Weaver, Ph.D. Candidate in the Department of Biology (Cram Research Group). In general, the pDONR221 vector (Invitrogen), which allows a target gene to be inserted into a variety of expression vectors using the Gateway LR recombination reaction, was used as an intermediate. The desired genes were first amplified from *C. roseus* RNA by qPCR and recombined with pDONR221 (Invitrogen, Carlsbad, CA) at the attB attachment sites to make pDONR221-*gene-of-interest* using the Gateway recombination. These pDONR221 constructs were then recombined into the pER8 vector (a gift from Prof. Chua,
Rockefeller University) using the available restriction sites in pER8. The silencing constructs contained a sense and anti-sense sequence separated by a short plant intron.

3.15.3. Electroporation of recombinant plasmids into A. rhizogenes

*Agrobacterium rhizogenes* is a soil bacterium that has the ability to transfer DNA from its cells to plant cells and simultaneously produce hairy root disease. This property was therefore used as a vehicle for genetic transformation of *C. roseus* by creating an *A. rhizogenes* construct with our desired gene of interest. Glycerol stocks of *A. rhizogenes* R1000 cultures (ATCC) were streaked onto plates containing solid Lysogeny Broth (LB) media containing 10g/L tryptone (Sunrise Science Products), 5g/L Bacto™ yeast extract (BD), 10g/L NaCl (Fisher Scientific), 15g/L Bacto™ agar (BD), pH = 7.0. LB media is prepared by autoclaving for 30 min, and adding bacterial selection antibiotic (filter-sterilized) after the media is cool. These *A. rhizogenes* R1000 cultures were maintained at 26°C for 2-3 days. A single colony was then transferred to liquid LB media (3 mL) in sterile culture tubes (#99445-13, Fisher Scientific) which were incubated at 26°C for 3 days at 250 rpm (EXCELLA E24R Incubator Shaker, New Brunswick Scientific).

These *A. rhizogenes* cultures (3 mL) were then chilled on ice for 15 min followed by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in chilled sterile water (600µL). The centrifugation and washing steps were repeated three times, after which the cells were resuspended in 150µL of chilled 10% glycerol (v/v, EM Science). These cells (100µL) were then electroporated with 1µL of plasmid DNA (100-150 ng/µL) at 1.8 kV (Bio-Rad Gene Pulser™). The transformed *A. rhizogenes* cells were then resuspended in LB media (500µL) and incubated at 250 rpm and 26°C for approximately 1 h before plating (100µL) on solid LB media containing antibiotic for bacterial selection. The *A. rhizogenes* colonies containing the desired plasmid grew on the plates containing the selection
antibiotic within 3-7 days following inoculation. A single colony was then transferred to LB media (3mL) containing the bacterial selection antibiotic and grown for 3 days at 250 rpm and 26°C. Glycerol stocks were prepared by adding 1mL of the liquid culture to 333µL of 60% sterile glycerol in a freezer vial (Fisher Scientific) and stored at -80°C until it was used for transformation of the C. roseus seedlings.

3.15.4. Genetic transformation of C. roseus with A. rhizogenes to generate transgenic C. roseus hairy root cultures

Glycerol stocks of A. rhizogenes containing the desired recombinant plasmid were streaked out on LB plates containing bacterial selection antibiotic. These plates were incubated at 26°C for 2-3 days (Lab-line Instruments). A single colony was then transferred to liquid LB media (3mL) also containing bacterial selection antibiotic, and liquid cultures were incubated at 26°C for 3 days at 250 rpm (EXCELLA E24R Incubator Shaker, New Brunswick Scientific) to select for only the bacteria cells containing the plasmid of interest.

Then, ~6 week old C. roseus seedlings were used for transformation by pricking the seedling stem between the cotyledons and the first set of true leaves with modified tweezers that had been dipped into the A. rhizogenes liquid cultures. Infected seedlings were maintained in the dark for 24 h before they were transferred to a 16 h light/8 h dark cycle (24 Watt Daylight, 6400 K, Pegasus Associates Lighting).

Hairy roots emerged from the site of infection approximately 4-6 weeks after infection. When these hairy roots were longer than 5mm in length (~6-8 weeks after infection), the root mass was excised and placed on solid Gamborg’s media (30 g/L sucrose, 1.55 g/L Gamborg’s salts, 1 mL/L vitamins, pH adjusted to 5.7, 6 g/L agar) containing carbenicillin (100 mg/L) and timentin (150 mg/L), termed “elimination media”, to kill any remaining A. rhizogenes. The roots
were transferred (and inverted onto) to fresh elimination plates each week, for 2-3 weeks, until individual roots were long enough to excise (~1 inch). Once long enough, individual hairy roots were isolated and transferred to plates containing Gamborg’s solid media and hygromycin (5mg/L), termed “first selection media”, to select for cultures containing the desired construct. These roots were traced on the selection plates in order to monitor growth. After 1 week, roots that grew and therefore contained the transferred construct, were selected again on Gamborg’s solid media containing 15mg/L hygromycin (“second selection media”). Growth was again monitored, and hairy roots that grew on two rounds of selection were either maintained on “post-selection” Gamborg’s plates containing just 150mg/L timentin (since carbenicillin was harmful to the roots long-term), or transferred to liquid Gamborg’s media with 150mg/L timentin and propagated.
4.0 An efficient method for estrogen-inducible transgene expression in Catharanthus roseus hairy roots

Noreen F. Rizvi, Miglia Cornejo, Kassi Stein, Jessica Weaver, Erin J. Cram, Carolyn W. T. Lee-Parsons

Plant Cell Tissue Organ Culture
Received: 14 July 2014 / Accepted: 28 August 2014 / Published online: 6 September 2014
DOI 10.1007/s11240-014-0614-1

4.1. Introduction:

The Catharanthus roseus plant is the source of several pharmaceutically valuable terpenoid indole alkaloids (TIAs), including the two anticancer drugs, vincristine and vinblastine. These secondary metabolites are produced at extremely low levels (0.0002 wt%), making commercial production expensive (Noble 1990). It is not currently possible to use other host systems for production since the TIA pathway is complex and not fully characterized (van der Heijden et al. 2004). Therefore, whole plants or plant tissue cultures are currently the only feasible routes for producing these valuable TIAs. Methods such as media optimization and elicitation have increased TIA production, but genetic engineering of C. roseus is a promising strategy to further improve metabolic flux and TIA concentrations.

Several enzymes and transcription factors involved in alkaloid biosynthesis in C. roseus have already been over-expressed through Agrobacterium-mediated genetic transformation (Zarate and Verpoorte 2007; Zhao et al. 2013). Initial transgene expression strategies used the constitutive cauliflower mosaic virus 35S promoter to drive transgene expression. An inducible system, however, is beneficial for several reasons. The timing of transgene expression can be controlled and expressed at the desired developmental stage, the uninduced condition serves as the negative control, and deleterious effects associated with constitutive expression such as
growth retardation can be minimized.

A glucocorticoid-inducible promoter system has been established in *C. roseus* hairy roots and has high and tightly controlled induction with low basal expression levels (Hughes et al. 2002). This system has been used to overexpress green fluorescent protein (GFP) (Hughes et al. 2002), several TIA biosynthetic enzymes (Hong et al. 2006b; Hughes et al. 2004a; Hughes et al. 2004b; Peebles et al. 2011) and a transcription factor, ORCA3 (Peebles et al. 2009). The glucocorticoid-inducible system includes an artificial GVG transcription factor containing a yeast GAL4 DNA-binding domain, the Herpes VP16 activation domain, and the rat glucocorticoid receptor (Aoyama and Chua 1997). The presence of the synthetic glucocorticoid hormone, dexamethasone, activates the GVG transcription factor inducing the expression of the gene of interest. However, growth defects observed in *Arabidopsis* (Kang et al. 1999) and rice (Ouwerkerk et al. 2001) have been attributed to one or more components of the chimeric GVG transcription factor. Furthermore, the expression of defense-related genes (such as pathogenesis-related genes) are induced by the glucocorticoid-inducible system in *Arabidopsis* (Kang et al. 1999).

An ethanol-inducible system has also been characterized in *C. roseus* hairy roots (Peebles et al. 2007a). This system is based on the ethanol-inducible alc regulon from *Aspergillus nidulans*. In the presence of ethanol, ALCR binds to the alcA promoter to drive transgene expression (Felenbok 1991). This system exhibits low background expression and high inducibility in *C. roseus* with no observable growth defects (Peebles et al. 2007a) and has recently been used to overexpress the transcription factor, ORCA2, in hairy roots (Li et al. 2013). However, the alc system exhibits higher basal expression in *Arabidopsis* tissue cultures and *C. roseus* hairy root cultures, potentially due to plant-produced ethanol (Li et al. 2013; Roslan et al. 45
Additionally, the \textit{alc} system can be induced by ethanol vapor (Salter et al. 1998) resulting from sterilization of seeds or equipment.

While glucocorticoid- and ethanol-inducible systems have been characterized for \textit{C. roseus}, the availability of several inducible systems would be beneficial for differential control of multiple transgenes. Several plant systems have been transformed using an estrogen-inducible system containing an artificial XVE transcription factor made up of the DNA-binding domain of the bacterial repressor \textit{LexA}, the Herpes \textit{VP16} activation domain, and the carboxyl region of the human estrogen receptor (Zuo et al. 2000). The XVE transcription factor is only activated in the presence of 17\textbeta-estradiol, which then binds to the \textit{LexA} operator sequence to induce transgene expression. This estrogen-inducible system has shown tight regulation and high inducibility in \textit{Arabidopsis}, tobacco, and rice (Okuzaki et al. 2011; Zuo et al. 2000). Although the transactivating \textit{VP16} sequence is the same, the estrogen-inducible system did not elicit a defense response in \textit{Arabidopsis} nor cause growth retardation in \textit{Arabidopsis} or rice (Okuzaki et al. 2011; Zuo et al. 2000).

The estrogen-inducible system has been implemented in \textit{C. roseus} cell suspensions (Xu and Dong 2007) but not in hairy root cultures. Hairy root cultures, transformed by \textit{Agrobacterium rhizogenes}, exhibit fast growth (Bhadra et al. 1993), genetic and biochemical stability (Aird et al. 1988; Peebles et al. 2007b), and can be grown unlimitedly in hormone-free media. Furthermore, hairy roots are beneficial over cell suspensions because they can potentially accumulate higher levels of alkaloids (Kittipongpatana et al. 1998; Moreno-Valenzuela et al. 1998) and have the ability to produce compounds found in wild-type roots of the parent plant without the loss of concentration frequently observed with cell suspension cultures (Veena and Taylor 2007). In this paper, we report the establishment of transgenic \textit{C. roseus} hairy root
cultures with estrogen-inducible expression of GFP. GFP as a visual reporter allows for the characterization of the estrogen-inducible system and optimization of the Agrobacterium-mediated transformation in C. roseus hairy roots.
4.2. Materials and Methods

4.2.1. Assaying the effect of hygromycin on *C. roseus* hairy roots

In this paper, wild-type (WT) hairy root cultures refers to *C. roseus* hairy root cultures generated by transformation with *A. rhizogenes* strain 15834 without any additional plasmids (a gift from Dr. Jacqueline V. Shanks, Dr. Ka-Yiu San, and Dr. Christie Peebles). These hairy roots were maintained as previously described (Goklany et al. 2013).

The effect of hygromycin was investigated on both WT and hygromycin-resistant (*hygR*) hairy roots. Hairy root pieces (3 cm) were inoculated onto solid half-strength Gamborg’s media (30 g/L sucrose, 1.55 g/L Gamborg’s B-5 salts, 1 mL/L 1000X Gamborg’s vitamins, 6 g/L agar, pH = 5.7) containing varying concentrations of hygromycin (0-30 mg/L) and monitored for 21 days.

4.2.2. Assaying the effect of elimination antibiotics on *C. roseus* hairy roots

Three WT hairy root pieces (3 cm) were inoculated into sterile 125-mL flasks containing 25mL liquid half-strength Gamborg’s media (30 g/L sucrose, 1.55 g/L Gamborg’s B-5 salts, 1 mL/L 1000X Gamborg’s vitamins, pH = 5.7). Varying concentrations of carbenicillin (0-100 mg/L) and/or timentin (0-150 mg/L) were added into each culture and monitored for 21 days. Two replicates were monitored at each antibiotic treatment condition. Hairy root tissue was harvested on day 21 and fresh and dry weights were recorded.

4.2.3. *C. roseus* seedling growth

*C. roseus* seeds (cv. Vinca Little Bright Eye, NEseed) were sterilized and planted as previously described (Weaver et al. 2014). In short, *C. roseus* seeds were surface-sterilized using 80% (v/v) ethanol for 30 sec followed by 30% (v/v) bleach containing 0.1% (v/v) Triton X-100 for 5 min. The seeds were rinsed 5 times and then soaked in sterile water for 24 hrs prior to being planted 1
mm below the surface of ~80 mL sterile solid full-strength Gamborg’s media (3.1 g/L Gamborg’s B-5 salts, 1 mL/L 1000X Gamborg’s vitamins, 5 g/L agar, pH = 5.7) in sterile Magenta GA-7 boxes. Seedlings were grown in the dark for one week and then transferred to a 16 hr photoperiod for ~6 weeks prior to infection with *A. rhizogenes*. Sucrose (30 g/L) was originally added in the Gamborg’s media to promote seedling growth rate, but later removed to prevent *A. rhizogenes* from overgrowing the *C. roseus* seedlings.

4.2.4. Electroporation of pER8-GFP plasmid into *Agrobacterium rhizogenes*

The pER8-GFP plasmid was electroporated into *A. rhizogenes* R1000 as previously described (Weaver et al. 2014). Briefly, glycerol stocks of *A. rhizogenes* R1000 cultures (ATCC 43056) were streaked onto plates containing solid Yeast Malt media (YM; 0.4 g/L BactoTM yeast extract, 10 g/L D-mannitol, 0.1 g/L NaCl, 0.1 g/L MgSO4, 0.5 g/L K2HPO4•3H2O, 6 g/L Bacto™ agar, pH 7.0). The bacterial cultures were maintained at 26°C for 2-3 days. A single colony was then transferred to 3mL of liquid YM media in sterile 13x100 mm culture tubes and incubated at 26°C and 250 rpm for 3 days. These *A. rhizogenes* cultures were then chilled on ice for 15 min, then centrifuged at 4000 rpm at 4°C for 10 min. The supernatant was discarded and the cells were resuspended in 600µL chilled sterile water. The centrifugation and washing steps were repeated three times, after which the cells were resuspended in 150µL of chilled 10% glycerol. These electrocompetent cells (100µL) were then electroporated with 1µL of the pER8-GFP plasmid DNA (100-150 ng/µL; obtained from Dr. Nam-Hai Chua, The Rockefeller University) at 1.8 kV (Bio-Rad Gene PulserTM). Then 500µL Lysogeny Broth media (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH = 7.0) was added to the electroporation cuvette. Transformed *A. rhizogenes* cells were incubated at 250 rpm and 26°C for ~1 hr before plating onto YM media containing 100 mg/L spectinomycin (MP Biomedicals). After 3 days, a
single colony was transferred to 3 mL of YM media containing 100 mg/L spectinomycin and grown for 2-3 days at 250 rpm and 26°C. Glycerol stocks were prepared by adding 1mL of the liquid culture to 333µL of 60% sterile glycerol and stored at -80°C.

4.2.5. Transformation of *C. roseus* seedlings with *A. rhizogenes*

Glycerol stocks of *A. rhizogenes* cells containing the pER8-GFP plasmid were streaked out on YM plates containing 100 mg/L spectinomycin for bacterial selection and incubated at 26°C for 2-3 days. A single bacterial colony was then transferred to liquid YM media (3mL) containing 100 mg/L spectinomycin and incubated at 26°C and 250 rpm for 3 days until the OD$_{600}$ = 0.5.

Six-week old *C. roseus* seedlings were transformed by pricking the seedling stem (between the cotyledons and the first set of true leaves) with modified tweezers that were dipped into the *A. rhizogenes* liquid cultures (OD$_{600}$ = 0.5). Infected seedlings were kept in the dark for 24 hrs before being maintained at a 16 hr photoperiod.

Hairy roots emerged from the site of infection approximately ~3 weeks after infection. When hairy roots were longer than 5 mm (~6 weeks after infection), hairy root clusters were excised along with a small piece of the seedling stem and placed on solid half-strength Gamborg’s media containing 100 mg/L carbenicillin (PhytoTechnology Laboratories) and 150 mg/L timentin (PhytoTechnology Laboratories) to kill remaining *A. rhizogenes*. Root clusters were subcultured onto fresh plates each week. After 2-3 weeks on *Agrobacterium*-elimination plates, individual hairy roots were transferred to solid half-strength Gamborg’s media containing 5 mg/L hygromycin (PhytoTechnology Laboratories) and 150 mg/L timentin for one week to select for transgenic root cultures. This concentration of hygromycin was chosen since our work shows that 2.5 mg/L hygromycin is toxic to WT hairy roots. To ensure hygromycin resistance, roots growing on 5 mg/L hygromycin and 150 mg/L timentin for one week were selected again.
on 15 mg/L hygromycin and 150 mg/L timentin for one week. Hairy root cultures that grew on both rounds of selection were transferred to 50mL liquid half-strength Gamborg’s media with 150 mg/L timentin for 5 subcultures before omitting timentin. Hairy root cultures were subcultured every 28-days and maintained as previously reported (Goklany et al. 2013).

4.2.6. Root induction and microscopy
Transgenic *C. roseus* hairy root cultures were analyzed for GFP expression using confocal microscopy. To induce GFP expression, 5µM 17β-estradiol (Sigma Aldrich) was used (Zuo et al. 2000). Root tips approximately 3 cm in length were aseptically excised from liquid-grown cultures and placed in six well plates with 5 mL filter-sterilized liquid half-strength Gamborg’s media with and without 5µM 17β-estradiol. The induced and uninduced root pieces were the same age and care was taken to ensure the root piece was approximately the same tissue size. The plates were incubated on an orbital shaker at 100 rpm and 26°C for 1 and 5 days (Hughes et al. 2002).

Images were obtained with a laser scanning confocal microscope (LSM 710, Carl Zeiss Microscopy GmbH) using an inverted microscope (Observer.Z1, Zeiss) and a 10X (NA 0.3) EC Plan-Neofluor objective. GFP was excited at a wavelength of 488 nm using a DPSS laser, and detected at 530nm.

4.2.7. Genomic DNA extraction and PCR
Frozen *C. roseus* root tissue was ground using a mortar and pestle, and 200 mg of ground tissue was added to 500µL of CTAB buffer (0.2 g/L CTAB, 1 mL/L 1 M Tris pH 8.0, 0.4 mL/L 0.5 M EDTA pH 8.0, 2.8 mL/L 5 M NaCl, 0.1 g/L PVP 40, pH = 5.0) in a microcentrifuge tube. The lysate was incubated for 15 min at 65°C. After incubation, the lysate was centrifuged at 12,000 rpm for 10 min to spin down cell debris. The supernatant was transferred to a clean
microcentrifuge tube. To the supernatant, 250µL of chloroform:isoamyl alcohol (24:1) was added and mixed by inversion. The samples were then centrifuged at 13,000 rpm for 2 min, after which the upper aqueous phase containing the DNA was transferred to a new microcentrifuge tube. To each sample, 50µL of 7.5 M ammonium acetate followed by 500µL of ice-cold absolute ethanol was added. The samples were incubated at -20°C for 20 min to precipitate the DNA. Following precipitation, the samples were centrifuged at 13,000 rpm and 4°C for 3 min. The supernatant was discarded, and the DNA pellet was washed twice with 500 µL of ice-cold 70% ethanol. After each wash, the DNA was centrifuged at 13,000 rpm and 4°C for 2 min. The supernatant was removed, and the DNA pellet was dried for approximately 5 min before it was resuspended in sterile DNase-free water. RNaseA (10 µg/ml) was added to the DNA to remove any remaining RNA. After resuspension, the DNA was incubated at 65°C for 20 min to destroy any DNases, and stored at 4°C.

PCR was used to amplify specific genes from the gDNA using primers designed for Rps9 (the housekeeping gene), Gfp, hygR, virD, and rolC (Table 4.9). The thermocycler protocol consisted of a heating step at 95°C for 10 min, then 30 cycles of 95°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min. After 30 cycles, the extension step at 72°C was repeated for another 10 min. PCR products were run on a 2% agarose gel and viewed under a UV transilluminator to verify product sizes. The products were extracted from the gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation) and sequenced to confirm the formation of the correct product (GENEWIZ Boston).
Table 4.9: Primer designs for Rps9, Gfp, hygR, virD2, and rolC. *primer sequences adapted from Haas et al. (1995), **primer sequences adapted from Suttipanta et al. (2011).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>Rps9 _F</td>
<td>TCCACCATGCCAGAGTGCTCATTA</td>
</tr>
<tr>
<td>Rps9 _R</td>
<td>TCCATCACCACCAGATGCCTTCTT</td>
</tr>
<tr>
<td>Gfp _F</td>
<td>GCCAACACTTGACTACTC</td>
</tr>
<tr>
<td>Gfp _R</td>
<td>CCTTAAGCTCGATCCTGTG</td>
</tr>
<tr>
<td>hygR _F187</td>
<td>GTTTATCGGCACCTTTGAC</td>
</tr>
<tr>
<td>hygR _R447</td>
<td>TCACGCCATGTAGTGTATG</td>
</tr>
<tr>
<td>virD2 _F1*</td>
<td>ATGCCGATCGAGCTCAAGT</td>
</tr>
<tr>
<td>virD2 _R388*</td>
<td>CCTGACCCAAACATCTCGGCTGCCC</td>
</tr>
<tr>
<td>RolC _F**</td>
<td>CAACCTGTTTCCTACTTTGTAA</td>
</tr>
<tr>
<td>RolC _R**</td>
<td>AAACAAGTGACACACTCAGCTTC</td>
</tr>
</tbody>
</table>

4.2.8. Gene expression analysis by qPCR

Hygromycin resistant transgenic *C. roseus* hairy root cultures were monitored for transcript levels of *Gfp* and defense-related genes. Roots were grown in liquid half-strength Gamborg’s media and induced with 5 µM 17β-estradiol on day 21 for 1 and 24 hrs.

mRNA was extracted from hairy root cultures using the RNAzol®RT (Molecular Research Center) method and quantified using a NanoDrop (ND-1000 Spectrophotometer; ThermoScientific). Extracted RNA was treated with DNase to remove genomic DNA, and cDNA was synthesized from the mRNA (1-5 µg; Deoxyribonuclease I Amplification Grade, SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen).

Besides *Rps9* and *Gfp*, the defense-related genes *Orca2*, *Orca3*, *Zct1*, *Zct2*, and *Zct3* were monitored using primer designs previously described (Goklany et al. 2013). Preparation for qPCR was performed using the RT² Real-TimeTM SYBR Green/Fluorescein PCR master mix (SABiosciences). qPCR was performed (iQ5 Real-Time PCR, Bio-Rad) using the thermocycler.
protocol previously described (Goklany et al. 2013). The amplification efficiency for each gene was calculated using Ct values over a range of cDNA dilutions and was ~100% for each gene monitored. Fold changes were calculated using the ΔΔCt method.
4.3. Results and Discussion

4.3.1. Low levels of hygromycin needed to select for transgenic *C. roseus* hairy roots

Selection of transformed tissue is a critical step in the development of transgenic plant cultures. The sensitivity of plant tissue to the selective agent can vary greatly with species and tissue type (Meng et al. 2007). An optimal concentration just above growth inhibition of non-transformed tissue can greatly improve selection efficiency without compromising selection against non-transformed tissue.

Kanamycin is commonly used as a selective agent, however, high levels of the compound are needed to inhibit growth. Because lower concentrations do not completely inhibit the growth of nontransgenic tissue, 100 mg/L kanamycin media is commonly used to select for transformed *C. roseus* hairy roots (Magnotta et al. 2007; Runguphan et al. 2009; Suttipanta et al. 2011; Zhou et al. 2010; Zhou et al. 2012). A major drawback of kanamycin is that many species show a high level of natural tolerance to this antibiotic, which can result in many untransformed escapers (Wilmink and Dons 1993). Additionally, regeneration from transformed rice calli was unsuccessful if kanamycin was used as the selective agent (Wilmink and Dons 1993). This is an important consideration since plant regeneration from transformed *C. roseus* tissue is a desirable downstream application (Choi 2004).

Hygromycin at concentrations ranging between 10-30 mg/L hygromycin have been previously used for selection in *Agrobacterium*-mediated *C. roseus* hairy root transformations (Hong et al. 2006b; Hughes et al. 2002; Hughes et al. 2004a; Hughes et al. 2004b; Peebles et al. 2009; Peebles et al. 2011; Wang et al. 2010a). However, no detailed study on the optimal hygromycin selection concentration for *C. roseus* hairy roots is available.

Therefore, hygromycin-resistant (*hygR*) and wild-type (WT, generated with *A. rhizogenes*
strain 15834 without any additional plasmids) roots were used to determine the optimal selection concentration of hygromycin. The WT roots (3 roots on the right side of each plate) demonstrate that 2.5 mg/L hygromycin is toxic and abolishes growth of WT roots lacking the hygR gene, whereas the hygR roots (3 roots on the left side of each plate) are able to detoxify the hygromycin and continue to grow on media containing up to 30 mg/L hygromycin (Figure 4.11).

![Figure 4.11: The effect of hygromycin on hygromycin-resistant (hygR) and wild-type (WT) hairy roots. hygR and WT C. roseus hairy roots were subjected to varying hygromycin concentrations (0-30 mg/L). Blue and black tracings represent hygR and WT root length at day 0. Visual differences between hygR and WT roots were apparent after 7 days, images were taken after 21 days of growth.](image)

Therefore, 5 mg/L hygromycin is an optimal concentration and was used for selecting transgenic hairy roots generated through Agrobacterium-mediated transformation. Additionally, the hygromycin concentration was gradually increased during the course of selection. Roots were selected on 5 mg/L hygromycin for one week, followed by 15 mg/L hygromycin for one week.
This step-wise selection helps further eliminate non-transgenic escapers, and the increase in concentration selects for transgenic lines strongly expressing hygR while giving the roots time to adapt to selection pressure. Furthermore, it has been shown that repeated selection may decrease growth but can help obtain high alkaloid-producing hairy root lines (Yukimune et al. 1994). Determining the optimal selective hygromycin concentration and using a stepwise selection is useful since unusually high and abrupt selection pressure may be harmful and inhibit growth of the transgenic cells and plants (Meng et al. 2007). Additionally, lower hygromycin levels are more cost-effective in selecting transgenic over non-transgenic roots.

**4.3.2. Elimination antibiotic carbenicillin has a negative effect on C. roseus hairy roots**

An ideal antibiotic for inhibiting Agrobacterium after genetic transformation should be highly effective, have no negative effect on plant growth and regeneration, and be inexpensive. Cefotaxime is one of the most commonly used antibiotics to eliminate the Agrobacterium from plant material after Agrobacterium-mediated plant transformations. Cefotaxime concentrations of 250 – 400 mg/L have been applied to C. roseus hairy roots to eliminate the Agrobacterium after infection (Bhadra et al. 1993; Runguphan et al. 2009; Zhou et al. 2012). However, A. rhizogenes strain R1000 was not used in these protocols. Although a higher cefotaxime concentration (400 mg/L) has been used in a few cases to eliminate A. rhizogenes strain R1000 (Magnotta et al. 2007; Suttipanta et al. 2011), high levels of cefotaxime (500 mg/L) have inhibited regeneration of Arabidopsis, snapdragon, wild carrot, apple, and white spruce, among others (Nauerby et al. 1997). We found that moderate levels of cefotaxime (250 mg/L) did not completely eliminate the R1000 strain after transformation. Therefore, other antibiotics commonly used against Agrobacterium were explored.

Carbenicillin and timentin are also commonly used to eliminate Agrobacterium after
plant transformations. Although high dosages of carbenicillin (250-1000 mg/L) had negative effects on *Arabidopsis*, snapdragon, beet, and tobacco among others (Nauerby et al. 1997), the combination of timentin and a lower concentration of carbenicillin has been effective in eliminating *Agrobacterium* (Cheng et al. 1998). Therefore, we used a combination of 100 mg/L carbenicillin and 150 mg/L timentin (in solid media) to eliminate R1000 strain immediately after roots were excised from the plant. The roots were transferred to fresh antibiotic-containing media each week for 2-3 weeks until they were long enough to individually isolate and select. The R1000 strain did not return in a majority of these root cultures, and no adverse effect of 100 mg/L carbenicillin and 150 mg/L timentin on hairy root cultures growing on solid elimination media was observed.

After the roots were selected on hygromycin-containing media (supplemented with 150 mg/L timentin), transgenic roots lines were transferred to liquid media. Although 100 mg/L carbenicillin and 150 mg/L timentin in the solid media has no negative effect on roots, the addition of these antibiotics (as low as 50 mg/L carbenicillin and 75 mg/L timentin) in liquid stunts growth dramatically (Figure 4.12). The root cultures show severe clumping and morphological defects. The fresh and dry weights of the tissue, however, are not affected (data not shown).

While the combination of carbenicillin and timentin produced growth defects in liquid media, the addition of timentin alone (up to 150 mg/L) did not (Figure 4.13). Carbenicillin has been shown to inhibit regeneration, inhibit rooting, and induce callus production in tobacco, thus timentin has been preferred over carbenicillin (Nauerby et al. 1997). Therefore, we used 150 mg/L timentin alone to eliminate the presence of residual R1000 strain in liquid cultures. Eliminating carbenicillin does not increase the return of the *Agrobacterium* in liquid cultures,
likely because the hairy root cultures have already been treated aggressively with carbenicillin and timentin on solid media.

<table>
<thead>
<tr>
<th>Antibiotic concentration (mg/L)</th>
</tr>
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<tbody>
<tr>
<td>Timentin: 0</td>
</tr>
<tr>
<td>Carbenicillin: 0</td>
</tr>
</tbody>
</table>

Figure 4.12: The effect of carbenicillin (0-100 mg/L) and timentin (0-150 mg/L) on *C. roseus* hairy root cultures in liquid media after 21 days.

<table>
<thead>
<tr>
<th>Antibiotic concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timentin: 0</td>
</tr>
<tr>
<td>Carbenicillin: 0</td>
</tr>
</tbody>
</table>

Figure 4.13: The effect of timentin alone (0-150 mg/L) on *C. roseus* hairy root cultures in liquid media after 21 days.
4.3.3. Stable GFP-expressing *C. roseus* hairy roots are established under control of an estrogen-inducible system

The estrogen-inducible expression system (XVE) has not been previously tested in *C. roseus* hairy roots. Using the optimized antibiotic selection and *Agrobacterium* elimination parameters for *Agrobacterium*-mediated transformations, we established estrogen-inducible *C. roseus* hairy roots expressing GFP under estrogen-inducible control with high efficiency (33%, n=234).

Transgenic hairy root lines were established using the *A. rhizogenes* R1000 strain containing the pER8-GFP construct (plasmid: 12,202 bp; T-DNA region: 5,692 bp) (Zuo et al. 2000). This construct encodes estradiol-inducible GFP expression (modified GFP which corrects for the silent mutation in plant cells (Kost et al. 1998; Zuo et al. 2000)) and confers hygR for selection. The R1000 strain has the chromosomal background of the *A. tumefaciens* strain C58 into which the Ri plasmid (pRiA4b) has been conjugated (White et al. 1985). In comparison, the commonly used strain 15834 harbors three large plasmids (pAr15834a, pAr15834b, and pAr15834c) but only one of these plasmids (pAr15834b) is needed for virulence (White and Nester 1980). Although both strains can induce the formation of hairy roots in *C. roseus* (Ciau-Uitz et al. 1994), 15834 exhibited the lowest infection rate and fewest number of roots compared to other *Agrobacterium* strains in other plant species (Lee et al. 2010; Vanhala 1995). Furthermore, difficulty eliminating excess bacterial growth from the 15834 roots has been previously reported (Vanhala 1995).

Upon transformation with R1000, 450 individual roots were isolated from seedlings, of which 234 roots grew on 5 mg/L hygromycin selection. These 234 roots were then selected on 15 mg/L hygromycin, of which 147 roots grew robustly. The overall efficiency after two selections was 33% (Table 4.10). This protocol results in comparable efficiencies to that
previously reported for *Agrobacterium*-mediated transformations of *C. roseus* hairy roots (16 – 27% with GVG inducible expression system, Hughes et al 2002, 2004a, 2004b; 50 – 60% with 35S constitutive system, Magnotta et al 2007; Wang et al 2010).

<table>
<thead>
<tr>
<th>Total # roots tested</th>
<th># roots surviving first selection (5 mg/L hygromycin)</th>
<th># roots surviving second selection (15 mg/L hygromycin)</th>
<th>Overall Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>450</td>
<td>234</td>
<td>147</td>
<td>32.7%</td>
</tr>
</tbody>
</table>

Table 4.10: Overall transformation efficiency of *C. roseus* hairy roots expressing GFP after two rounds of hygromycin selection.

Of the 147 lines surviving two rounds of selection on hygromycin, inducible GFP expression of 75 lines was verified (remaining 72 lines were not checked). The concentration of 5 µM 17β-estradiol saturates the GFP signal in transgenic *Arabidopsis* plants (Zuo et al. 2000) and is the minimum concentration for homogenous GFP signals in transgenic rice calli (Okuzaki et al. 2011). Therefore, *C. roseus* root tips were treated with 5 µM 17β-estradiol and viewed using confocal microscopy 24 hr after induction alongside uninduced root tips of each line. Of the 75 lines, 47 lines (62.7%) show strong GFP expression and 28 lines (37.3%) show weak or patchy expression. This data confirms the high selection efficiency of hygromycin since there was 100% correlation between hygromycin-resistance and GFP expression in transgenic roots, although expression levels were variable among different lines.

Additionally, the transgenic lines demonstrate low basal expression levels. Of the 75 lines checked for GFP expression, 72 lines (96%) have no expression while 3 lines (4%) show some GFP expression in the absence of 17β-estradiol. This could be due to positional effects resulting from random insertion of the T-DNA into the *C. roseus* genome. Similarly, in the glucocorticoid-inducible system, transgenic *C. roseus* root tips showed nonspecific GFP florescence (Hughes et
al. 2002), and 6.5% of transgenic rice lines showed leaky GUS expression (Ouwerkerk et al. 2001) in the absence of dexamethasone. Unlike our *C. roseus* lines, the estrogen-inducible system in rice calli did not show any leaky expression (Okuzaki et al. 2011).

GFP expression in *C. roseus* hairy roots varies with tissue type. GFP levels are highest in the fast-growing meristem regions such as the root tips (Figure 4.14, A-C), which is similar to *C. roseus* GFP lines under control of the glucocorticoid-inducible system (Hughes et al. 2002). The center, more mature regions of the roots show weaker expression (Figure 4.14, D-F). This is likely due to the tissue being older and thicker, therefore less penetrable by the estradiol.

Figure 4.14: Inducible GFP expression in transgenic *C. roseus* hairy roots. Root tips were induced with 5μM 17B-estradiol for 24 hrs and viewed under a laser scanning confocal microscope. The region of the root in which GFP expression was observed is shown on the right.
GFP expression is sustained in roots induced with 5 µM 17β-estradiol for five days (Figure 4.15). The modified GFP in the pER8-GFP construct has a half-life of ~18 hrs (de Ruijter et al. 2003). Therefore, either the estrogen-inducible system continues to induce Gfp expression over several days, or the Gfp transcript is relatively stable and continues to be translated to produce new GFP protein. Similar findings were reported in the glucocorticoid- and ethanol-inducible systems (induction sustained for 100 and 72 hrs, respectively) in C. roseus (Hughes et al. 2002; Peebles et al. 2007a).

Of the 47 lines showing strong GFP expression, 26 robustly growing lines were adapted to liquid media, of which 9 have survived six or more subcultures. The 17 lines that did not survive the adaptation to liquid either did not make it past the second subculture (14/17) or were lost to Agrobacterium contamination (3/17). The adaptation to liquid media has been shown to be a difficult step in C. roseus hairy root development, and often reduces the number of viable transgenic lines (Bhadra et al. 1993).

![Figure 4.15: Sustained GFP expression in transgenic C. roseus hairy roots. Root tips were induced with 5uM 17B-estradiol and viewed using a laser scanning confocal microscope after 1 and 5 days.](image)
4.3.4. Transgenes are stably integrated into genome of transgenic hairy roots

Insertion of the transgenes was confirmed by PCR in 15 of the 26 transgenic lines adapted to liquid media (remaining 11 lines were not checked). Genomic DNA (gDNA) was extracted from each line and PCR was used to amplify *Rps9*, *Gfp*, *hygR*, *virD2*, and *rolC* genes (Figure 4.16).

All 15 lines show amplification of *Rps9*, the housekeeping gene, verifying successful extraction of gDNA. The *rol* genes (*rolA*, *rolB*, and *rolC*) are transferred *A. rhizogenes* genes essential for the initiation of hairy roots (Veena and Taylor 2007). The *rolC* gene was monitored and is present in each of the lines, ensuring these are hairy roots generated through *A. rhizogenes*-mediated transformation. *Gfp* and *hygR*, genes in the transferred T-DNA region, are amplified in all lines except in the WT line, as expected. 100% (all 15) of the transgenic lines contain both the *hygR* and *Gfp* genes, supporting the conclusion that both genes were integrated simultaneously into the plant genome during T-DNA transfer. This is important since the T-DNA region can be truncated in *Agrobacterium*-mediated transformations.

The absence of a virulence gene (*virD2*) exclusive to *A. rhizogenes* was also checked to ensure that the *Gfp* and *hygR* genes were amplified from the *C. roseus* genome and not from *A. rhizogenes* contamination in the hairy root cultures. The virulence (*vir*) genes are *Agrobacterium* genes necessary for T-DNA processing and transfer. Specifically, *virD2* is an endonuclease responsible for cleaving the T-DNA borders before transfer (Haas et al. 1995). None of the 15 transgenic lines or the WT line contain *virD2*. 
Figure 4.16: Genomic integration of transgenes in transgenic *C. roseus* hairy roots. *Rps9*, *Gfp*, *hygR*, *virD2*, and *rolC* genomic DNA was amplified in 15 different transgenic lines by PCR. WT = wild-type hairy roots, R1000 = *A. rhizogenes* containing pER8-GFP plasmid, NT = no template control.

### 4.3.5. *Gfp* expression in transgenic hairy roots is strongly and rapidly inducible

*Gfp* transcript levels were monitored in three transgenic lines. Well-growing lines 1, 10, and 29 were induced with 5 µM 17β-estradiol on day 21 for 0 (untreated), 1, and 24 hrs. RNA was extracted and cDNA was synthesized using the oligo-dT primers, excluding any contaminating bacterial RNA. *Gfp* expression in all three transgenic lines is very low in the absence of 17β-estradiol (untreated condition). As expected, *Gfp* expression levels in WT roots treated with 17β-estradiol are undetectable (data not shown). Upon 17β-estradiol treatment, *Gfp* expression in all three transgenic lines increases dramatically and significantly by 140-fold and 1730-fold after 1 hr and 24 hrs compared to untreated controls (Figure 4.17).
4.3.6. Estrogen-inducible XVE system does not elicit a defense response

The biosynthesis of TIAs in *C. roseus* is associated with the pathogen defense response. Jasmonate, produced in response to pathogen attack, regulates TIA biosynthesis through several transcription factors including ORCA (Octadecanoid-responsive *Catharanthus* AP2-domain) and ZCT (Zinc finger) proteins (Memelink et al. 2001; Menke et al. 1999a; Pauw et al. 2004; van der Fits and Memelink 2000). Previously, we demonstrated that a transient *Agrobacterium*-mediated expression method induced ORCA and ZCT in *C. roseus*, complicating its application for studying TIA regulation (Weaver et al. 2014). Therefore, the effect of the estrogen-inducible system on the induction of defense-related genes (*Zct* and *Orca*) was monitored in the transgenic GFP-expressing *C. roseus* hairy root lines.

Figure 4.17: *Gfp* transcript levels in transgenic GFP-expressing *C. roseus* hairy root cultures. Transgenic hairy root cultures were treated with 5uM 17B-estradiol for 0 (untreated), 1 and 24 hrs. Expression of *Gfp* was monitored by qPCR for three individual transgenic lines. Expression levels were normalized relative to *Rps9*, and fold differences were calculated relative to the untreated condition by the ddCt method. A) Fold increases in *Gfp* expression in individual lines. Error bars denote standard deviations of qPCR triplicates. B) Average *Gfp* fold increases in three lines. Error bars denote standard deviations of three lines. Significance for the estradiol-treated conditions was calculated relative to the untreated condition using the Student’s t-test (* denotes p < 0.05).
Three GFP-expressing hairy root lines (1, 10, and 29) were induced with 5 µM 17β-estradiol on day 21 for 0 (untreated), 1, and 24 hrs. qPCR was used to monitor Zct1, Zct2, Zct3, Orca2, and Orca3 expression (Figure 4.18). Compared to the untreated condition, average Zct2, Zct3, and Orca3 expression levels do not increase significantly upon induction with 17β-estradiol after 1 or 24 hrs. Expression levels of Zct1 increase significantly (1.9-fold) after 1 hr with estradiol treatment. However, Zct1 expression levels in WT roots are much more strongly induced by 250 µM MeJA (8.6-fold) (Rizvi et al. 2013). Levels of Orca2 significantly decrease (0.5-fold) 24 hrs after 17β-estradiol treatment (Figure 4.18). In comparison, Orca2 levels increased significantly (29-fold) in WT roots when induced with 250 µM MeJA (Rizvi et al. 2013). Therefore, although the changes in Zct1 and Orca2 expression are significant with 17β-estradiol treatment, the magnitude of change is much smaller than seen with MeJA induction. The small changes in gene expression may be attributable to differences in root tissue growth between flasks. The WT roots treated with 17β-estradiol did not show any significant changes in expression of Zct or Orca.

These results suggest that estradiol and the XVE system in transgenic C. roseus hairy roots do not induce the expression of defense-related transcription factors. In contrast, the activation of GVG in the glucocorticoid-inducible system induced defense-related genes in Arabidopsis (Kang et al. 1999), making it difficult to distinguish between specific changes from a particular transgene and changes resulting from an activated GVG inducible system. The estrogen-based inducible system, however, does not exhibit these effects in the stable line and therefore is applicable for studying the defense response in C. roseus.
Figure 4.18: *Zct1*, *Zct2*, *Zct3*, *Orca2*, and *Orca3* expression in transgenic GFP-expressing and WT *C. roseus* hairy root cultures. Expression of *Zct* and *Orca* transcripts were monitored by qPCR for three individual transgenic lines and the WT line. Expression levels were normalized relative to *Rps9*, and fold differences were calculated relative to the respective untreated cultures by the ddCt method. Error bars denote standard deviations of three lines. Significance for the estradiol-treated conditions was calculated relative to the untreated condition using the Student’s t-test (* denotes $p < 0.05$).
4.4. Conclusions

Here, we present an effective protocol for Agrobacterium-mediated transformation and generation of transgenic *C. roseus* hairy roots with estrogen-inducible GFP expression (Figure 4.19). This protocol, including selection dosage and antibiotics used to eliminate residual *A. rhizogenes* R1000, resulted in a transformation efficiency of 33%.

The estrogen-inducible system provides advantages as an additional inducible system for manipulating and understanding the regulation of TIA biosynthesis in *C. roseus*. For instance, the estrogen-inducible system exhibits low basal expression, rapid and sustained induction of the transgene, with no apparent adverse effects on *C. roseus* hairy roots. Importantly, the estrogen-inducible system does not elicit ORCA and ZCT expression involved in the defense response of *C. roseus*. These characteristics are ideal in an expression system for studying the regulation of TIA biosynthesis and for increasing the production of these compounds.

1) Grow *C. roseus* seedlings for 4 – 6 weeks in sucrose-free media under a 16 hr photoperiod.
2) Infect seedlings using modified tweezers covered in *A. rhizogenes* R1000.
3) After ~6 weeks, excise roots onto solid media containing carbenicillin (100 mg/L) and timentin (150 mg/L) for 1 week to eliminate *A. rhizogenes*. Repeat until individual roots are long enough to isolate.
4) Isolate individual roots and select transgenics using 5 mg/L hygromycin (+ 150 mg/L timentin) for 1 week followed by 15 mg/L hygromycin (+150 mg/L timentin) for 1 week
5) Adapt transgenic hairy root culture to liquid media containing 150 mg/L timentin. Confirm genomic integration of transgenic lines.

*Figure 4.19: Summary of optimized Agrobacterium-mediated transformation method for the generation of C. roseus hairy roots.*
4.5. Author contributions

NFR generated the transgenic cultures, conducted all experiments and wrote the initial version of this manuscript. MC and KS assisted NFR with generating the transgenics and with most of the experiments. JW, EJC, and CLP were all involved in troubleshooting experiments and revising the manuscript. All authors read and approved the manuscript.

4.6. Acknowledgements

This work was supported by the National Science Foundation (NSF CBET Award #1033889).

The authors thank Dr. Nam-Hai Chua (The Rockefeller University, New York) for providing the pER8-GFP vector and Dr. Jacqueline V. Shanks (Iowa State University, Ames, IA), Dr. Ka-Yiu San (Rice University, Houston, TX) for providing the wild-type *C. roseus* hairy root cultures.
5.0 Silencing the repressor, ZCT1, does not further increase terpenoid indole alkaloid levels in methyl-jasmonate elicited C. roseus hairy roots

5.1. Introduction

*Catharanthus roseus* is the source of many valuable terpenoid indole alkaloids (TIAs), including the anticancer compounds vinblastine and vincristine. Despite the low production levels of these compounds (0.0002 wt%) in *C. roseus*, these pharmaceuticals are effective and continue to be used for cancer treatments (van der Heijden et al. 2004). The structural complexity and complicated biosynthetic pathway of TIAs prohibits their chemical synthesis or production in host systems (Zhao et al. 2013), but makes them ideal targets for improved production through metabolic engineering. *C. roseus* hairy root cultures have become an ideal system for studying the production of TIAs due to their genetic and biochemical stability, and fast growth in hormone-free media.

TIAs are formed in *C. roseus* from the condensation of two precursor pathways. Tryptamine from the indole pathway and secologanin from the terpenoid pathway condense to form strictosidine, the backbone of TIAs (Figure 5.20). Initial precursor feeding analysis suggested that TIA production is likely limited by either precursor, tryptamine or secologanin (Goklany et al. 2009; Lee-Parsons and Royce 2006; Morgan and Shanks 2000; Whitmer et al. 1998; Whitmer et al. 2002a; Whitmer et al. 2002b). Therefore, early genetic engineering manipulations focused on overexpressing biosynthetic enzymes in those precursor branches, particularly the first committed steps of each branch (Table 5.11). Genetic manipulations of the indole pathway, either single gene or combined genes (such as *Asa*, *Tdc*, *Asa + Tdc*, *Asaβ*, or *Asaβ + Tdc*) effectively increased the TIA precursor, tryptamine, but did not lead to large
increases in downstream TIAs. Similarly, overexpression of key enzymes in the terpenoid pathway (such as $Dxs$ and $G10h$) did not significantly increase TIA levels.

Instead of manipulating biosynthetic genes, transcription factors (TFs) are promising metabolic engineering targets due to their ability to regulate multiple biosynthetic pathway genes. Therefore, recent efforts to increase TIA levels in $C.~roseus$ have focused on the transcriptional regulation of TIAs through TFs (Memelink and Gantet 2007). Jasmonate (or methyl jasmonate, MJ), a phytohormone produced in a defense response, signals a cascade of transcription factors that regulate TIA biosynthetic enzymes. These include the transcriptional activators ORCA2, ORCA3, BPF1, MYC1, MYC2, and WRKY1 and the transcriptional repressors JAZ, ZCT1, ZCT2, ZCT3, GBF1 and GBF2. Our research focuses on the ORCA and ZCT transcription factors since they are downstream TFs and respond to MJ, whereas other downstream TFs, such as WRKY1 and GBF do not (Goklany et al. 2013).

The ORCA (octadecanoid-responsive $Catharanthus$ AP2/ERF domain) transcription factors, including ORCA1, ORCA2, and ORCA3, are well-known transcriptional activators of several biosynthetic genes in $C.~roseus$ (Figure 5.20). However, when $Orca3$ was overexpressed in $C.~roseus$ hairy roots, TIA levels did not significantly increase despite increased expression of several biosynthetic genes such as $Asa$, $Dxs$, $Str$, and $Sls$ (Peebles et al. 2009). Overexpression of $Orca2$ increased levels of tryptamine, ajmalicine, serpentine, catharanthine, 16-hydroxytabersonine and 19-hydroxytabersonine, but decreased levels of tabersonine, strictosidine and horhammericine; these metabolite changes were associated with an increase in $Asa$, $Tdc$, $G10h$, $Str$, $T16h$, and $D4h$ (Li et al. 2013). $Orca1$ is not induced by MJ and only minimally activated the $Str$ promoter (Menke et al. 1999a), therefore has not been extensively studied.
Despite the increases in biosynthetic gene expression upon Orca overexpression, elicitation with JA alone had a larger increase in TIA levels (Peebles et al. 2009) since several biosynthetic genes are not regulated by ORCA (van der Fits and Memelink 2000). For example, levels of Tdc and G10h were not affected by Orca3 overexpression and levels of Sgd decreased (Peebles et al. 2009). Similarly, levels of Dxs and 16Omt were unaffected by Orca2 overexpression and levels of Sgd and Dat decreased (Li et al. 2013). Other transcriptional activators are likely involved in the MJ-induced expression of these TIA genes.

Three members of the Cys_{2}/His_{2}-type (transcription factor IIIA-type) zinc finger protein family, ZCT1, ZCT2, and ZCT3, are transcriptional repressors that repress the activity of TDC and STR (Pauw et al. 2004). In addition, the ZCT proteins are able to counteract transcriptional activation of Tdc and Str by ORCAs (Pauw et al. 2004). In C. roseus hairy roots, MJ induces both Orca and Zct, and relatively high levels of Zct, especially Zct1, are correlated with repressed TIA levels (Goklany et al. 2013). Furthermore, overexpression of Orca2 (Li et al. 2013) or Orca3 (Peebles et al. 2009; Zhou et al. 2010) either directly or indirectly induces the expression of Zct (this was confirmed by Weaver et al., in submission). Therefore our strategy involves elicitation with MJ to induce TIA genes through TFs (such as ORCAs) while simultaneously silencing ZCT1 to counter its activation by MJ and ORCA.

In this study, we explore the effect of silencing Zct1 in C. roseus hairy roots elicited with MJ. An estradiol-inducible system was used to successfully control timing of Zct1 silencing. The effect on TIA production and the expression of TIA biosynthetic genes was analyzed. The expression of other transcription factors was also analyzed to further understand the regulation of TIAs in C. roseus.
Figure 5.20: Terpenoid indole alkaloid (TIA) biosynthesis in *C. roseus*. Solid arrows indicate single step, whereas dashed arrows represent multi-step enzymatic conversions. Enzymes activated by ORCA3 and/or repressed by ZCT1 (based on binding and correlation studies) are indicated by a green arrow or red stop, respectively.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>TIA biosynthetic gene(s) overexpressed</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indole pathway</strong></td>
<td>Feedback-insensitive <em>Asα</em></td>
<td>* 300-fold increase in tryptophan</td>
<td>(Hughes et al. 2004a)</td>
</tr>
<tr>
<td></td>
<td>* 10-fold increase in tryptamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* 1.7-fold increase of lochnericine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*no significant increase in tryptamine</td>
<td></td>
<td>(Hughes et al. 2004b)</td>
</tr>
<tr>
<td></td>
<td>* 1.3-fold increase in serpentine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feedback-insensitive <em>Asα + Tdc</em></td>
<td>* 6-fold increase in tryptamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>* no increase in downstream TIA s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feedback-insensitive <em>Asα + Asβ</em></td>
<td>* 40-fold increase in tryptophan</td>
<td>(Hong et al. 2006a)</td>
</tr>
<tr>
<td></td>
<td>* 8-fold increase in tryptamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* varied results on TIA levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feedback-insensitive *Asα + Asβ + Tdc</td>
<td>* 14-fold increase in tryptophan</td>
<td></td>
</tr>
<tr>
<td></td>
<td>* 3-fold increase in tryptamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Terpenoid pathway</strong></td>
<td><em>Dxs</em></td>
<td>* 1.7-fold increase in levels of ajmalicine and lochnericine</td>
<td>(Peebles et al. 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* decreased levels of tabersonine and horhammericine</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Dxs and G10h</em></td>
<td>* 1.4-fold increase in tabersonine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 1.2-fold increase in lochnericine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* overall positive gain in metabolites compared to the mixed results of</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>overexpressing DXS alone</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Terpenoid and indole pathways</strong></td>
<td>* 1.2-fold increase in tabersonine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Dxs and feedback-resistant <em>Asα</em></td>
<td>* 1.2-fold increase in lochnericine</td>
<td></td>
</tr>
<tr>
<td><strong>Downstream</strong></td>
<td><em>Dat</em></td>
<td>* 4-fold increase in levels of horhammericine</td>
<td>(Magnotta et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* no change in levels of other TIA s</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.11: Genetic engineering efforts to overexpress single or multiple TIA biosynthetic enzymes in *C. roseus* hairy roots. *Asα* = anthranilate synthase α subunit; *Asβ* = anthranilate synthase β subunit; TDC = tryptophan decarboxylase; DXS = 1-deoxy-D-xylulose-synthase; G10H = geraniol-10-hydroxylase; DAT = deacetylvindoline 4-O-acetyltransferase
5.2. Materials and Methods

5.2.1. Preparation of pER8-Zct1hp and pER8-GFP hp plasmids and electroporation into Agrobacterium rhizogenes

pER8-GFP and pSK-Int were obtained from Dr. Nam-Hai Chua (The Rockefeller University). The pSK-Int vector is an intermediate cloning plasmid for generating hairpin RNAi constructs. It contains the third intron from the Arabidopsis actin-11 gene with a multiple cloning site (MCS) on each side of the intron. Two 163bp fragments of Zct1 (Genbank accession AJ632082) were amplified from C. roseus cDNA using primers containing restriction sites and cloned into each of the MCSs to generate a hairpin in the pSK-Int vector (Table 5.12). The Zct1-hairpin was subsequently removed from pSK-Int and cloned into the pER8 backbone using restriction cloning (XhoI-SpeI). A GFP-hairpin construct in pUC57(kan) was synthesized by Genewiz containing the same actin-11 intron from pSK-Int. A SpeI site and XhoI site flanks the hairpin construct on opposite sides (Table 5.12). The GFP-hairpin construct was moved from pUC57(kan) to pER8 using restriction cloning.

DH5α Escherichia coli competent cells were used for cloning (Z-competent E. coli Transformation Kit, G-Biosciences). E. coli was grown in LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl with 15 g/L agar for plates, pH= 7.0) at 37°C overnight at 250 rpm when in liquid culture. The pER8-Zct1hp and pER8-GFP hp constructs were electroporated into Agrobacterium rhizogenes R1000 (ATCC 43056) as previously described, except LB media was used instead of YM (Rizvi et al. 2014). R1000 was grown at 26°C for 2-3 days at 250 rpm when in liquid culture. See Table 5.12 for antibiotic resistance conferred by each vector and concentrations used.
### Primers for cloning Zct1 in MCS1 of pSK-Int

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zct1FXhol</td>
<td>TAACTCGAGGGCGGAGATCTACTCCATCAATCTTTAGCGGTGACGAAGCCGAAAACTCAT</td>
</tr>
<tr>
<td>Zct1RHindIII</td>
<td>TAAAGGCTTATACCTCACACCAACCCGGGTTCCTCTTCACCGAAGTCAT</td>
</tr>
</tbody>
</table>

### Primers for cloning Zct1 into MCS2 of pSK-Int

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zct1FSpeI</td>
<td>TAAACTAGTGCCGAGATCTACTCCATCAATCTTTAGCGGTGACGAAGCCGAAAACTCAT</td>
</tr>
<tr>
<td>Zct1RecoRI</td>
<td>TAAGAATTCATACCTCACCACCACCACGGGTTCCTCTTCACCGAAGTCAT</td>
</tr>
</tbody>
</table>

### Zct1 transcript and sequence used (red) in Zct1-hairpin (5’ to 3’)

ATTCCACCTAATTTAAAAATTTATATCTAATCTAAATTCCTCAAGTGAATGGTTTCATCAATCTTTAGCGGTGACGAAGCCGAAAACTCAT
ATGAGACCGCCACCGGCGGCCCCTCTGAGTGAGAGAAACCGGTTGGTGGTGTAGT
GATAAAACCTTGATCGAACGGCATACCATTCCTGATGAGATCTAATAGCAAGTAAAGG
GGTTTTTGAGATTTGAGTACGCCGAGTTAGTACGTCATTGGAGCTCCTCGAGCAACGA
TGTTCTATATAGTGCGCTTCGCAATTTTCCATTAGGCCTGCTTTCGAGGGTCAT
ATGAGACCGCCACCGGCGGCCCCTCTGAGTGAGAGAAACCGGTTGGTGGTGTAGT
GATAAAACCTTGATCGAACGGCATACCATTCCTGATGAGATCTAATAGCAAGTAAAGG

### GFP Sequence used in GFP-hairpin (5’ to 3’)

GTGAAGGTGATGCAACATACGGAAAACTTACCTAAAAATTTATTTATTTGACTACTGGA
AAAACCTTGATCGAACGGCATACCATTCCTGATCCTACCGGCCTCGAGTGA
CTTCTCCCGTTACCCTGATCATATGAAAGCGGACGACTTACTTTCAAGAGCGCCA
Table 5.12: Primers used for cloning Zct1-hairpin into pER8, Zct1- and GFP-hairpin sequences, pER8-Zct1hp plasmid map and antibiotic resistance conferred by each plasmid.
5.2.2. Transformation of *C. roseus* seedlings with *A. rhizogenes*

*C. roseus* seedlings were grown aseptically and transformed with *A. rhizogenes* R1000 containing pER8-Zct1hp, as previously described (Rizvi et al. 2014). Of the 428 individual roots tested, 144 survived two rounds of selection on hygromycin for an overall transformation efficiency of 33.6% (Table 5.15). Of these selected lines, 26 well-growing lines were moved to liquid media, and 8 survived long-term subculture in liquid (over 16 subcultures by time of publication). These transgenic hairy root cultures were subcultured every 28-days and maintained as previously reported (Goklany et al. 2013).

5.2.3. Genomic DNA extraction and PCR

Genomic DNA was extracted from transgenic *C. roseus* roots using the CTAB method as previously described (Rizvi et al. 2014). PCR was used to amplify specific genes from the gDNA using primers designed for *Rps9* (the housekeeping gene), *LexA*, *hygR*, *virD*, and *rolC* (Table 5.13). The thermocycler protocol consisted of a heating step at 95°C for 10 min, then 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min. After 30 cycles, the extension step at 72°C was repeated for another 10 min. PCR products were run on a 2% agarose gel and viewed under a UV transilluminator to verify product sizes. The products were extracted from the gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation) and sequenced to confirm the correct product (GENEWIZ, Boston).
Table 5.13: Primer sequences used to check genomic integration. *primer sequences adapted from (Haas et al. 1995). **primer sequences adapted from (Suttipanta et al. 2011).

5.2.4. Induction of transgenic hairy roots with estradiol and/or MJ

To express the silencing hairpin (Zct1hp or GFPhp), transgenic *C. roseus* hairy roots were induced with 5µM 17β-estradiol (Fisher Scientific) on day 26 (late-exponential growth phase). Previously, induction with 5µM 17β-estradiol showed strong and tightly regulated expression of GFP in control transgenic *C. roseus* hairy roots (Rizvi et al. 2014). Stock solutions (5mM) of 17β-estradiol were prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich), and 50µL of stock solution was added to 50mL of culture media to achieve a final concentration of 5µM 17β-estradiol. Uninduced cultured were treated with 50µL of DMSO.

Cultures were elicited with MJ (≥ 95%, Sigma Aldrich) 24 h after induction with 17β-estradiol. Stock solutions were prepared in ethanol (200 proof, ACS/USP grade, Pharmco-AAPER) and added to 50mL of culture media to achieve final concentrations of 250µM or 1000µM MJ. Cultures were harvested 8, 24, and 48 h after MJ addition for mRNA analysis or
after 3, 5, and 7 days for TIA metabolite analysis. *C. roseus* hairy roots were blotted and flash-frozen in LN₂.

### 5.2.5. Extraction of TIA metabolites from *C. roseus* hairy root cultures

TIAs were extracted from transgenic *C. roseus* hairy root cultures as previously described (Goklany et al. 2013). In short, frozen hairy root cultures were lyophilized using a Flexi-Dry MP Freeze-Dryer (Kinetics Thermal Systems). Root cultures were pulverized using a mortar and pestle and ~50mg of dried root powder was extracted using 5mL of methanol (HPLC grade) twice. The extracts were pooled and concentrated overnight (Savant SpeedVac Plus Concentrator, Thermoquest). The dried alkaloid-containing extracts were then re-dissolved in 1mL of methanol (HPLC grade) and filter-sterilized using non-sterile syringe filters (Millipore Millex Nonsterile Syringe Filters) into HPLC vials (Waters Corp.).

### 5.2.6. TIA metabolite analysis by HPLC

TIA levels in the *C. roseus* hairy root extracts were analyzed through HPLC (Waters 2695 Separations Module, Waters 996 Photodiode Array Detector, Empower 2 Software) and separated using a reversed-phase C18 column (Luna, 150 x 4.60 mm ID column, 5 mm particle size, Phenomenex).

The HPLC mobile phases for TIA separation were: 99.9% water with 0.1% (v/v) formic acid as the aqueous phase, and 99.9% acetonitrile with 0.1% (v/v) formic acid as the organic phase. The protocol consisted of the following steps: 1) 90% aqueous and 10% organic as the initial condition, 2) gradient to 70% aqueous and 30% organic over 20 minutes, 3) gradient to 100% organic over 8 minutes, 4) gradient to 90% aqueous and 10% organic over 10 minutes, and 5) isocratically at 90% aqueous and 10% organic for 20 minutes to equilibrate the column for the next injection. All the flow rates were maintained at 1.0 mL/min.
Strictosidine (274 nm), ajmalicine (254 nm), serpentine (254 nm), tabersonine-like peak 5 (329 nm), tabersonine-like peak 6 (329 nm), and tabersonine (329 nm) were monitored at the respective wavelengths and verified through pure standards or Beer’s Law correlations. MS analysis was previously performed to verify the known compounds (Goklany et al. 2009).

**5.2.7. RNA extraction and gene expression analysis by qPCR**

Transcript levels of transcription factor and TIA biosynthetic genes were monitored in the transgenic *C. roseus* hairy root cultures by qPCR. mRNA was extracted from frozen hairy root cultures using the RNAzol®RT (Molecular Research Center) method and quantified using a NanoDrop (ND-1000 Spectrophotometer; ThermoScientific). Extracted RNA was treated with DNase to remove genomic DNA, and cDNA was synthesized from the mRNA (1-5 µg; Deoxyribonuclease I Amplification Grade, SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen).

Transcription factor genes (*Orca2, Orca3, Zct1, Zct2*, and *Zct3*) and TIA biosynthetic genes (*Tdc, Str*, and *G10h*) were monitored using the primers listed in Table 5.14. qPCR was performed using the RT² Real-Time™ SYBR Green/ROX PCR master mix (SABiosciences) and QuantStudio 6 (Applied Biosystems) using the thermocycler protocol previously described (Goklany et al. 2013). The amplification efficiency for each gene was calculated using Ct values over a range of cDNA dilutions and was ~100% for each gene monitored. Fold changes were calculated using the ∆∆Ct method.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orca2_F</td>
<td>GAAATTCGCTGCGGAAATCAGGGA</td>
</tr>
<tr>
<td>Orca2_R</td>
<td>AGATGACACGATGAAGATCGGCGT</td>
</tr>
<tr>
<td>Orca3_F</td>
<td>TGTCAGGAGGATTCTGTGTGTGGGA</td>
</tr>
<tr>
<td>Orca3_R</td>
<td>CGCATATTAAACCGGCTGCATCA</td>
</tr>
<tr>
<td>Zct1_F</td>
<td>AATCTTTTAGCGGTACGAAGCAGGA</td>
</tr>
<tr>
<td>Zct1_R</td>
<td>CGTTGTCTCACGGCCTCAATTC</td>
</tr>
<tr>
<td>Zct2_F</td>
<td>TTTCCATCTGTTTCAAGCTCTCCG</td>
</tr>
<tr>
<td>Zct2_R</td>
<td>ATTACCCGACGCGCAATCCTCTCAT</td>
</tr>
<tr>
<td>Zct3_F</td>
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<td>Zct3_R</td>
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</tr>
<tr>
<td>Tdc_F</td>
<td>ACCTACGACGTCGAAACGGATT</td>
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<td>Tdc_R</td>
<td>AAACCTCGGACATATACAGGCGCAT</td>
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<tr>
<td>Str_F</td>
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<tr>
<td>Str_R</td>
<td>GGTGTTGGAAGTGTATAAA</td>
</tr>
<tr>
<td>G10h_F</td>
<td>TAGCAGGGACGGACACAACATCAA</td>
</tr>
<tr>
<td>G10h_R</td>
<td>TCACGTCCAATTGCCCCAAGGCATTC</td>
</tr>
</tbody>
</table>

Table 5.14: Primer sequences used for qPCR analysis of *C. roseus* transcription factor and TIA biosynthetic genes. *Orca* and *Zct* primers previously described in (Goklany et al. 2013). *Tdc* and *G10h* primers previously described in (Goklany et al. 2009). *Str* primers are newly designed.
5.3. Results and Discussion

5.3.1. Transgenic hairy root cultures exhibit low levels of Zct1 upon induction

The estrogen-inducible system has been shown to be a tightly regulated and highly inducible system in C. roseus hairy roots (Rizvi et al. 2014). Therefore, we established transgenic C. roseus hairy root cultures with estrogen-inducible expression of the Zct1-hairpin to induce RNA interference (referred to as Zct1hp in this text). Additionally, C. roseus hairy roots with estrogen-inducible silencing of green fluorescent protein (GFP) were established as a control for the effects of RNAi (referred to as GFPhp in this text). Of the 428 Zct1hp roots generated, 144 survived two rounds of selection on hygromycin-containing media, resulting in a transformation efficiency of 33.6% (Table 5.15). Similarly, 255 GFPhp roots were generated, of which 71 survived two rounds of selection, resulting in a transformation efficiency of 27.8%. These efficiencies are comparable to previously reported efficiencies using this optimized Agrobacterium-mediated transformation method and estrogen-inducible construct (Rizvi et al. 2014).

<table>
<thead>
<tr>
<th>Line</th>
<th>Total # roots tested</th>
<th># roots surviving first selection (5mg/L hygromycin)</th>
<th># roots surviving second selection (15mg/L hygromycin)</th>
<th>Overall Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zct1hp</td>
<td>428</td>
<td>215</td>
<td>144</td>
<td>33.6%</td>
</tr>
<tr>
<td>GFPhp</td>
<td>255</td>
<td>117</td>
<td>71</td>
<td>27.8%</td>
</tr>
</tbody>
</table>

Table 5.15: Overall transformation efficiency of transgenic Zct1hp and GFPhp C. roseus hairy roots after two rounds of hygromycin selection

Of these 144 transgenic Zct1hp lines, 26 well-growing lines were originally adapted to liquid culture, of which 8 lines survived long-term subculture in liquid (over 16 subcultures at the time of publication). Of the 71 transgenic GFPhp lines, 36 were originally adapted to liquid culture, of which 10 have survived long-term subculture in liquid (3-5 subcultures at the time of publication).
publication). The adaptation of cultures to liquid is reportedly a difficult and limiting step in generating transgenic *C. roseus* hairy roots (Bhadra et al. 1993).

From the transgenic lines originally transferred to liquid culture, integration of the transgenes was verified in 10 Zct1hp lines and 9 GFPhp lines (remaining lines not checked; Figure 5.21). All transgenic Zct1hp and GFPhp lines have *Rps9* (housekeeping gene), *LexA* (promoter for the chimeric XVE transcription factor, part of the estrogen-inducible construct), *hygR* (hygromycin-resistance gene), and *rolC* (hairy root control). None of the lines (except faint band in Zct1hp-12) have *virD2* (*Agrobacterium* specific virulence gene), which indicates the successful elimination of *Agrobacterium* and confirms the transgenes are not due to any contaminating *Agrobacterium*. 
Figure 5.21: Genomic integration verified in transgenic *C. roseus* hairy roots. *Rps9* (housekeeping gene), *LexA, hygR, virD2* (*Agrobacterium* control), and *rolC* (hairy root control) genomic DNA was amplified in 10 Zct1hp and 9 GFPhp transgenic lines by PCR. WT = wild-type hairy roots, R1000 = *A. rhizogenes* containing pER8-Zct1hp or pER8-GFPhp plasmid, NT = no template control.

Levels of Zct1-silencing under estradiol induction were assessed in transgenic *C. roseus* hairy root cultures through qPCR. 17β-estradiol (5µM) was added to three individual Zct1hp lines (Zct1hp-36, Zct1hp-38, Zct1hp-40) for 24 h since estrogen-inducible gene expression is high at this concentration and timepoint (Rizvi et al. 2014). Zct1-silencing was highly variable between the three estradiol-induced Zct1hp lines ranging from 0.3- to 0.9-fold decrease compared to their untreated controls (Figure 5.22). For example, Zct1hp-38 has strong silencing
(0.3-fold), but Zct1hp-36 is barely silenced (0.9-fold) upon induction with 17β-estradiol. The variability in Zct1-silencing might be attributed to gene copy number and/or positional effects associated with the random T-DNA integration of Agrobacterium-mediated transformations.

Neither 17β-estradiol nor any elements of the artificial transcription factor, XVE, alters expression of Zct1 (Rizvi et al. 2014). Furthermore, Zct1 expression levels are not significantly affected by 17β-estradiol in the GFPhp controls, indicating that the induction of RNAi does not induce Zct1 expression (Figure 5.22). All three Zct1hp lines, representing the range of low to high Zct1-silencing, were investigated for TIA production and expression of TIA genes and transcriptional regulators.

![Figure 5.22: Zct1 expression in individual Zct1hp-36, Zct1hp-38, Zct1hp-40, and GFPhp-29 lines with 5μM 17β-estradiol treatment for 24 h. Fold change is calculated with respect to each line’s untreated control. Error bars represent standard deviations of qPCR triplicates.](image-url)
5.3.2. Zct1 induction by MJ is abolished in Zct1-silenced lines

MJ induces TIA production through the action of several transcriptional activators (MYC2, ORCA2, and ORCA3) (van der Fits and Memelink 2000; Zhang 2011). But MJ also induces the expression of the ZCT transcriptional repressors (Goklany et al. 2013; Pauw et al. 2004; van der Fits and Memelink 2000). TIA production is high with 250µM MJ at which there is a high Orca to low Zct ratio, while TIA production is repressed at 1000µM MJ which induces high Zct to low Orca levels (Goklany et al. 2013). Therefore, we examined whether Zct1 was effectively silenced with estradiol at both low and high MJ dosages. 17β-estradiol (5µM) was first added to Zct1hp lines for 24 h, followed by the addition of either 250µM or 1000µM MJ for 8, 24 and 48 h. Zct1 expression was monitored through qPCR.

Induction with 17β-estradiol alone successfully decreases expression of Zct1 in line Zct1hp-38, and this decrease is sustained over 48 h (Figure 5.23). Zct1 levels increase moderately upon elicitation with 250µM MJ and strongly with elicitation with 1000µM MJ in Zct1hp-38, similar to that previously reported (Goklany et al. 2013). However, upon treatment with 17β-estradiol and 250µM MJ, the increase of Zct1 by MJ is completely abolished (Figure 5.23). Similarly, treatment with 17β-estradiol and 1000µM MJ significantly reduces the induction of Zct1. This indicates that Zct1 silencing can effectively knock-down the induction of Zct1hp-38 hairy roots by MJ. This effect is also seen in lines Zct1hp-40 and Zct1hp-36 to a lesser degree (Figure 5.24). Importantly, in the GFPhp-29 control line, Zct1 expression is not significantly changed upon induction with 17β-estradiol alone or in the presence of 250µM MJ (Figure 5.24).
Figure 5.23: Zct1 expression in Zct1hp-38 over 48 h. 5µM 17β-estradiol was added for 24 h, then 250µM MJ or 1000µM MJ was added for the time specified. Error bars represent standard deviations of qPCR triplicate.
Figure 5.24: Zct1 expression in Zct1hp-36 Zct1hp-40 and GFPhp-29 over 48 h. 5µM 17β-estradiol was added for 24 h, then 250µM MJ was added for the time specified. Error bars represent standard deviations of qPCR triplicates.

5.3.3. MJ-elicited TIA production does not further increase upon Zct1-silencing

Since Zct1 levels were successfully and significantly decreased upon induction with 17β-estradiol, the effect of Zct1-silencing on MJ-elicited TIA production was analyzed. Zct1hp lines were treated with 5µM 17β-estradiol for 24 h, followed by 250µM MJ for 5 d. Levels of metabolites were monitored in the hairy root lines that were checked for silencing (Zct1hp-36, Zct1hp-38, and Zct1hp-40), as well as four additional lines with confirmed genomic integration of the Zct1-silencing construct (Figure 5.25).
None of the metabolites are affected by treatment with 17β-estradiol alone, suggesting the important role of elicitors, such as MJ, in inducing transcriptional activators and TIA production. Levels of several metabolites, including strictosidine \((p = 0.1)\), tabersonine \((p = 0.08)\), and tabersonine-like compounds (peak 5 and 6, \(p = 0.05)\) increase with 250µM MJ induction as expected, but there is no further increase when induced with both 17β-estradiol and 250µM MJ (Figure 5.25). When GFPhp lines were analyzed for TIA metabolite levels, the trends were consistent: MJ induced levels of some metabolites but 17β-estradiol alone or with MJ had no effect (data not shown). Interestingly, Zct1hp lines that show strong silencing (Zct1hp-38 and Zct1hp-40) also show this overall trend, but levels of Zct1hp-36, with low Zct1-silencing, shows higher levels of certain metabolites (Figure 5.25). Metabolite levels between the highest (Zct1hp-44 and Zct1hp-36) and lowest (Zct1hp-21) producing lines varied by 10-fold. These results, taken together, indicate that there is strong variability between lines, but overall, the effect of Zct1-silencing on TIA production was not more significant than the addition of MJ alone.

In the literature, levels of specific metabolites increased with overexpression efforts: ajmalicine levels increased upon ORCA2 or DXS and G10H overexpression, (Li et al. 2013; Peebles et al. 2011), serpentine levels increased upon either TDC, ORCA2 or WRKY1 overexpression (Hughes et al. 2004b; Li et al. 2013; Suttipanta et al. 2011), and tabersonine increased with DXS+G10h, DXS+ASα overexpression (Peebles et al. 2011). The overall flux through the TIA biosynthetic pathway, however, has not increased through the genetic engineering of a single or combination of two enzymes/transcription factors. In this study, we see that the overall flux is largely responsive to MJ treatment, but the silencing of Zct1 does not further increase the metabolic flux. Elicitation with MJ dominating the effects of genetic engineering has been seen before (Peebles et al. 2009). This work further emphasizes the need
for better understanding of the mechanisms and identifying the regulatory network by which MJ induces TIA gene and metabolite production.

Figure 5.25: Average TIA metabolite levels of seven Zct1hp lines (Zct1hp-21, -36, -38, -40, -42, -43, -44). Estradiol (5µM) was added for 24 h, then 250μM MJ was added for 5 days. Bars represent the mean and standard deviations of the seven lines.
When Zct1hp roots were compared to wild-type (WT) roots, new metabolites were identified. In addition to an increase in strictosidine, tabersonine and tabersonine-like compounds upon induction with 250µM MJ, the Zct1hp metabolite profile indicates the presence of new metabolites observed at 329nm (Figure 5.26). Two peaks, one at 13.5 min (potentially 19-acetyltabersonine with echitovenine based on MS/MS analysis; termed “peak 3”) and one at ~16 min (unknown compound with MW = 393.183 and/or 395.198 based on MS/MS analysis; termed “peak 6”) are present in Zct1hp hairy roots but not WT roots, even in the presence of MJ. The UV spectrum for both of these new compounds is similar to tabersonine. These peaks are only present upon MJ induction, and do not further increase with 17β-estradiol and MJ, indicating the compounds are not associated with Zct1-silencing. This was confirmed in the GFPhp control hairy roots: both peaks 3 and 6 are produced in the presence of MJ, irrespective of 17β-estradiol.

The discrepancy in metabolites between the Zct1hp/GFPhp and WT line is likely attributed to the genes associated with A. rhizogenes strain R1000 compared to the 15834 strain used to generate the WT hairy roots. One or a few T-DNA genes are often lost during transformation with A. rhizogenes, and the presence or absence of important genes in the T-DNA of A. rhizogenes affects TIA production (Taneja et al. 2010). The A. rhizogenes rol genes are T-DNA genes responsible for hairy root formation and have the potential to activate secondary metabolite production in plant cells (Bulgakov 2008). The rol genes can vary in size depending on the A. rhizogenes strain, and different rol genes are found in different A. rhizogenes strains (Veena and Taylor 2007). Although both 15834 and A4 are agropine-type strains, differences may arise due to the fact that R1000 has the chromosomal background of the A. tumefaciens strain C58 into which the root-inducing plasmid (pRiA4b) has been conjugated (White et al.
Furthermore, the loss of certain *rol* genes (or other poorly characterized T-DNA genes) during T-DNA transfer may be associated with *A. rhizogenes* strain and therefore contributing to new metabolites found in the *C. roseus* hairy root lines generated with R1000.

![HPLC chromatograms in Zct1hp, GFPhp and WT hairy root lines. Estradiol (5µM) was added for 24hrs, then 250µM MJ was added for 5 days. Blue line highlights peak 5 (13.5 mins) and red line highlights peak 6 (16 min), which are only present in the Zct1hp and GFPhp lines upon MJ treatment. Note: the exact dry weight extracted (~50mg) varied slightly between samples.](image)

**Figure 5.26:** HPLC chromatograms in Zct1hp, GFPhp and WT hairy root lines. Estradiol (5µM) was added for 24hrs, then 250µM MJ was added for 5 days. Blue line highlights peak 5 (13.5 mins) and red line highlights peak 6 (16 min), which are only present in the Zct1hp and GFPhp lines upon MJ treatment. Note: the exact dry weight extracted (~50mg) varied slightly between samples.

### 5.3.4. TIA metabolites inhibited upon 1000µM MJ induction, despite *Zct1*-silencing

TIA metabolite levels did not increase further upon *Zct1* silencing combined with 250µM MJ. However, *Zct1* levels are low at 250µM MJ relative to 1000µM MJ (2.5-fold compared to 27-fold, respectively; Figure 5.23). To investigate the effect of *Zct1*-silencing when MJ-induced *Zct* levels are high, we analyzed TIA metabolite levels in Zct1hp roots treated with 17β-estradiol and...
1000µM MJ. We showed that the Zct1-silenced lines are able to overcome the induction by 1000µM MJ (Figure 5.23) and would expect TIA levels to be affected. A representative transgenic hairy root line with considerable Zct1 silencing, Zct1hp-40, was treated with 5µM 17β-estradiol for 24 h, followed by 250µM MJ or 1000µM MJ addition for 3 and 7 d.

Levels of ajmalicine and serpentine do not increase upon induction with 1000µM MJ or 17β-estradiol and 1000µM MJ (Figure 5.27), similar to that observed with 250µM MJ. Upon induction with 1000µM MJ, levels of strictosidine, tabersonine, and tabersonine-like compounds (peak 5 and 6) are all significantly lower than at 250µM MJ induction; this inhibition at 1000µM MJ has been previously reported (Goklany et al. 2013). Surprisingly, levels of these TIAs still do not increase with 17β-estradiol and 1000µM MJ treatment, where Zct1 levels are low (Figure 5.27). This suggests that the mechanism by which TIA production is repressed at 1000µM MJ is not solely regulated by ZCT1.
Figure 5.27: TIA metabolite levels in Zct1hp-40. 17β-estradiol (5µM) was added for 24 h, then 250µM or 1000µM MJ was added for the time specified (3 and 7 d). Error bars represent standard deviations between two biological replicates.
5.3.5. TIA gene expression levels correlate with TIA production in Zct1-silenced lines

To understand why TIA levels did not further increase with Zct1-silencing, the expression of several TIA biosynthetic genes was analyzed. G10H and TDC catalyze the first committed steps of the terpenoid and indole pathways, respectively. Furthermore, ZCT is known to bind to and repress the expression of Tdc, Str (Pauw et al. 2004), and G10h (unpublished data). Therefore, the expression of G10h, Tdc, and Str was monitored by qPCR. 17β-estradiol (5µM) was added to Zct1hp-38 for 24 h, followed by 250µM or 1000µM MJ for 8, 24, and 48 h.

Although levels of Zct1 decrease, levels G10h, Tdc, and Str do not increase upon treatment with 17β-estradiol alone (Figure 5.28). Similar to TIA metabolite production, levels of G10h, Tdc, and Str increase upon treatment with 250µM MJ, but do not increase further upon treatment with 17β-estradiol and 250µM MJ (Figure 5.28). This trend is similarly observed in Zct1hp-36 and Zct1hp-40 lines (Figure 5.29). Silencing of Zct1 does not have a significant effect on MJ-elicited TIA gene expression. These results explain the lack of increase seen in TIA metabolite levels upon Zct1 silencing (Figure 5.25).

Interestingly, levels of G10h, Tdc, and Str are still elevated after 48 h (Figure 5.28), which differs from wild-type C. roseus hairy roots where TIA gene expression levels typically peak at 24 h and then decrease (Goklany et al. 2013). G10h, Tdc, and Str expression remains elevated in the GFPhp-29 control line (Figure 5.30), indicating the discrepancy between the silenced lines and wild-type line is likely attributed to the T-DNA genes associated with A. rhizogenes strain R1000 compared to the 15834 strain used to generate the wild-type hairy roots.

As previously observed (Goklany et al. 2013), expression levels of G10h, Tdc, and Str are inhibited in Zct1hp roots upon treatment with 1000µM MJ (Figure 5.28), and do not increase upon treatment with 17β-estradiol. While the overall lack of increase is surprising, TIA gene
expression levels are strongly correlated with TIA production. A lack of increase in TIA levels (Figure 5.27) can be explained by a lack of increase in TIA biosynthetic gene expression (Figure 5.28).

TIA gene expression is not increased upon Zct1-silencing of MJ-induced hairy roots either at low or high MJ dosages. Silencing Zct1 alone is not sufficient to increase the expression of genes that ZCT1 is known to repress, suggesting that other transcription factors induced by MJ contribute to the repression of TIAs.

Figure 5.28: G10h, Tdc, and Str expression in Zct1hp-38. 17β-estradiol (5μM) was added for 24 h, then 250μM or 1000μM MJ was added for the time specified (8, 24 and 48 h). Error bars represent standard deviations of qPCR triplicates.
Figure 5.29: *Tdc*, *Str* and *G10h* expression in lines Zct1hp-40 and Zct1hp-36. 17β-estradiol (5µM) was added for 24 h, then 250µM MJ was added for the time specified (8 and 24 h). Error bars represent standard deviations of qPCR triplicates.
Figure 5.30: Tdc, Str and G10h expression in GFPhp-29 line. 17β-estradiol (5µM) was added for 24 h, then 250µM MJ was added for the time specified. Error bars represent standard deviations of qPCR triplicates.
5.3.6. *Orca* levels are correlated with TIA metabolite levels

Although *Zct1* was successfully silenced, expression of TIA genes regulated by *Zct1* was unchanged (Figure 5.28). To investigate if other transcription factors are dominating or compensating for the effect of *Zct1*-silencing, the expression of other transcription factors in *C. roseus* were monitored. 17β-estradiol (5µM) was added to *Zct1*hp-38 for 24 h followed by the addition of 250µM or 1000µM MJ for 8, 24, and 48 h. *Orca2* and *Orca3*, transcription factors known to activate *Tdc* and *Str*, were monitored by qPCR. In addition, *Zct2* and *Zct3*, transcriptional repressors of *Tdc* and *Str*, were also monitored through qPCR.

In *Zct1*hp roots, levels of *Orca2* and *Orca3* are strongly induced with 250µM MJ but are significantly lower at 1000µM MJ, especially after 48 h (Figure 5.31). This trend with MJ dosage is consistent with the expression of TIA genes (*G10h, Tdc* and *Str*; Figure 5.28) and with TIA production (Figure 5.27). As expected, *Orca* expression was not affected by *Zct1*-silencing with 17β-estradiol, either alone or in the presence of MJ. When *Orca2* and *Orca3* levels were monitored in GFPhp-29 control roots, RNAi (17β-estradiol treatment) also had no affect on *Orca* expression (Figure 5.33). Levels of *Orca2* and *Orca3* in *Zct1*hp roots are also sustained after 48 h, similar to TIA gene expression. Hence, the elevated and sustained expression of *Orca* is not specific to *Zct1*-silencing but is consistent with TIA gene expression.

The expression of *Zct2* and *Zct3* was also analyzed in *Zct1*hp lines. Probably due to high homology and the RNAi mechanism, *Zct2* expression was also silenced, even in the presence of MJ (Figure 5.31). The silencing of *Zct2* was not as strong in other lines monitored (Figure 5.32). In contrast, levels of *Zct3* in *Zct1*hp-38 (and *Zct1*hp-36) remain high upon treatment with 17β-estradiol and MJ. When the induction of *Zct* was analyzed in the GFPhp control line, *Zct3* (but not *Zct1* or *Zct2*) is induced by 17β-estradiol and therefore activated by RNAi (Figure 5.33). The
induction by RNAi (~4-fold), however, is significantly lower than with MJ-treatment (11-15-fold).

In ORCA2- and ORCA3-overexpressing hairy roots, Zct1, Zct2 and Zct3 levels increased and the effects of overexpressing Orca alone could not be discerned from elevated levels of the Zct repressors (Li et al. 2013; Peebles et al. 2009). In this study, however, Orca levels remain high through MJ elicitation while Zct1 (and Zct2) levels remain low through silencing; yet TIA production was not significantly enhanced. ORCA and ZCT proteins bind to overlapping but distinct regions on the Str promoter, and activation by ORCA is counteracted by ZCTs (Pauw et al. 2004). The strong correlation between Orca levels, TIA gene expression and TIA production suggests that activation of TIA genes by ORCA plays an important role at these physiological conditions. However, basal levels of Zct (particularly Zct2 and Zct3, ΔCt = ~0) in hairy root cultures are more abundant at untreated conditions compared to Orca levels (particularly Orca3, ΔCt = -5) (Figure 5.34). Therefore, large fold-increases in Orca may not mean higher absolute transcript/protein levels, and protein levels should be checked. Western blotting methods using custom antibodies for ZCT1 and ORCA3 were unsuccessful in this study, likely due to the short duration of transcription factor proteins.

Although the three ZCT proteins are thought to be functionally similar in their repression of TIA genes such as Tdc and Str, they are structurally different. For example, ZCT1 and ZCT2 are both smaller (~20 kDa) than ZCT3 (27 kDa), which also has a longer spacer between its zinc finger motifs (Pauw et al. 2004). Recently, ZCT1 and ZCT2, but not ZCT3, was shown to repress the activity of hydroxymethylbutenyl 4-diphosphate synthase (HDS) in the monoterpenoid pathway (Chebbi et al. 2014). These factors suggest distinct functions of each of the ZCT transcriptional repressors. Since all three ZCT repressors were not silenced in any of the
lines analyzed, the non-silenced ZCT proteins may be repressing TIA genes in the absence of ZCT1. Alternatively, if the ZCT repressors, especially ZCT3, have distinct functions, then it would be important to silence of all three ZCT repressors simultaneously.

Figure 5.31: Orca2, Orca3 Zct2 and Zct3 expression in Zct1hp-38. 17β-estradiol (5µM) was added for 24 h, then 250µM or 1000µM MJ was added for the time specified (8, 24 and 48 h). Error bars represent standard deviations of qPCR triplicates.
Figure 5.32: *Orca2*, *Orca3* Zct2 and Zct3 expression in Zct1hp-40 and Zct1hp-36. 17β-estradiol (5µM) was added for 24 h, then 250µM or 1000µM MJ was added for the time specified (8 and 24 h). Error bars represent standard deviations of qPCR triplicates.

Figure 5.33: *Orca2*, *Orca3* Zct2 and Zct3 expression in GFP hp-29 hairy roots. 17β-estradiol (5µM) was added for 24 h, then 250µM or 1000µM MJ was added for the time specified (8 and 48 h). Error bars represent standard deviations of qPCR triplicates.
Figure 5.34: Basal levels of \textit{Zct1}, \textit{Zct2}, \textit{Zct3}, \textit{Orca2}, \textit{Orca3}, \textit{G10h}, \textit{Tdc} and \textit{Str} in untreated \textit{WT}, \textit{Zct1hp}, and \textit{GFP-overexpressing hairy roots}. * denotes $p<0.05$
5.4. Conclusions

In this study, we successfully developed transgenic *C. roseus* hairy roots with inducible Zct1-silencing (through RNAi). Upon induction with 17β-estradiol, levels of Zct1 significantly decrease compared to untreated controls. Importantly, the silencing of Zct1 abolishes Zct1 induction by MJ, at both low (250µM) and high (1000µM) concentrations. Despite these low levels of Zct1, MJ-elicited TIA production does not further increase in Zct1-silenced lines. This is particularly surprising at 1000µM MJ since the reduction of Zct1 expression is more substantial at that condition.

To understand why TIA levels did not further increase, TIA gene and TF expression were analyzed. MJ-elicited TIA gene expression (*G10h, Tdc* and *Str*) also does not further increase in Zct1-silenced cultures and is therefore strongly correlated with TIA production. Similarly, expression of *Orca2* and *Orca3* is strongly correlated with TIA gene expression and TIA production. Interestingly, due to high homology, levels of Zct2 (but not Zct3) are also silenced in Zct1-silenced lines, even under MJ-elicited conditions. The ZCT repressors are thought to play overlapping but distinct functions in *C. roseus*. Therefore, to eliminate TIA repression by other ZCTs, silencing the expression of all three ZCT repressors simultaneously could potentially result in increased TIA production.
5.5. Author contributions

NFR generated the transgenic cultures, conducted the analysis of the transgenics, and wrote the initial version of this manuscript. JW cloned the pER8-Zct1hp and generated the A. rhizogenes with each construct. JW, EJC, and CLP were all involved in troubleshooting experiments and revising the manuscript. All authors read and approved the manuscript.

5.6. Acknowledgements

This work was supported by National Science Foundation (NSF) CBET Award #1033889 to CLP and EJC and by the American Association of University Women (AAUW) Dissertation Fellowship to NFR. The authors thank Dr. Nam-Hai Chua (The Rockefeller University, New York) for providing the pER8 vector and also thank Kassi Stein and Caroline Webb for their assistance in generating the pER8-Zct1hp and pER8-GFPhp transgenic cultures.
6.0  **ZCT1 is a modulator between growth and defense in *C. roseus***

6.1. Introduction

The tradeoff between growth and defense in plants, thought to be due to resource restrictions, has been well documented for decades but is poorly understood (Huot et al. 2014; Staswick 1992). The plant hormones jasmonate (JA) and giberellin (GA) regulate defense and growth, respectively, and often antagonistically. Produced by the plant under stress or pathogen attack, JA promotes the interaction of COI1 protein with JAZ repressors, resulting in JAZ ubiquitination and degradation, thereby releasing MYC2 proteins to initiate a defense response (Chini et al. 2007; Thines et al. 2007). This co-receptor complex is central in JA-signaling in a variety of plants, including *Arabidopsis, Nicotiana tabacum, Catharanthus roseus*, and *Artemisia annua* (De Geyter 2012). In an analogous cascade, GA binds to GID1, which interacts with the DELLA family transcription factors (Sun 2011). The DELLA repressors are then ubiquitinated and degraded. Degradation of DELLA releases downstream transcription factors, such as the PIFs (Phytochrome Interacting Factor), thus activating growth-related responses (Sun 2011). DELLA proteins are highly conserved among different species, including *Arabidopsis*, barley, *Brassica*, grape, maize, rice, and wheat (Thomas and Sun 2004).

One mechanism by which defense is prioritized over growth in *Arabidopsis* was recently uncovered. DELLA proteins interact competitively with PIF and JAZ proteins. In the absence of GA, DELLA proteins sequester both PIF and JAZ into inactive complexes. PIF therefore cannot activate growth-related responses, and JAZ is unable to inhibit MYC2 which then promotes JA-related responses (Hou et al. 2010; Yang et al. 2012). Hence, defense is prioritized over growth. In the presence of GA, however, DELLA proteins are degraded, releasing PIF to promote growth.
and JAZ to bind and repress MYC2, thus attenuating JA-response (Hou et al. 2010; Yang et al. 2012). Here, growth-related responses are prioritized over defense. DELLA therefore mediates crosstalk between the GA and JA signaling pathways by protein-protein interactions with GA- and JA-associated transcription factors.

Since DELLA does not bind to DNA, an intermediate protein is needed that binds to both DELLA and the regulated gene. Recently, a class of INDETERMINATE DOMAIN (IDD) family proteins was identified in Arabidopsis (Feurtado et al. 2011; Yoshida et al. 2014). The IDD proteins can interact simultaneously with DNA and DELLA, unlike other reported DELLA-interaction partners (Yoshida et al. 2014). Transactivation studies showed IDDs alone possess only low transcriptional activation activity, but that IDDs function as intermediate proteins for the transcriptional activation of Arabidopsis genes by DELLA (Yoshida et al. 2014). Therefore, DELLAs positively regulate the expression of growth-associated genes through interaction with IDD proteins (Yoshida et al. 2014).

The medicinal plant, C. roseus, has been widely studied for the regulation of its defense-related secondary metabolites, such as anticancer compounds vinblastine and vincristine. Understanding the interplay between growth and defense in C. roseus could help elucidate the control and switch to production of desirable compounds. In C. roseus, JA induces the biosynthesis of defense-related terpenoid indole alkaloids (TIAs) through a network of many transcription factors including the JA-responsive COI1-JAZ-MYC2 cascade (Zhang 2011; Zhang 2008). JA-activated MYC2 induces the transcriptional activators, ORCAs, which induce the expression of genes encoding TIA biosynthetic enzymes (Memelink et al. 2001; Menke et al. 1999a; van der Fits and Memelink 2001). JA also induces the expression of transcriptional repressors, ZCT (Pauw et al. 2004). ZCT is strongly associated with defense in C. roseus; its
expression is induced not only by JA, but also by physical stress and *Agrobacterium* (Goklany et al. 2013; Weaver et al. 2014). However, silencing ZCT1 did not increase TIA production, despite ZCT1’s ability to bind and repress several TIA biosynthetic genes (Rizvi et al. In submission), suggesting it may play a more versatile role in overall plant metabolism.

In this work, we demonstrate that ZCT1 is also involved in growth and is activated by GA-signaling in *C. roseus* seedlings but not in hairy roots. In addition, we identify DELLA proteins in *C. roseus* and its interaction with PIF and JAZ, demonstrating this cascade in another species besides *Arabidopsis*. We also hypothesize that Zct1 expression is regulated by the DELLA and IDD complex to ultimately modulate growth in *C. roseus*. Understanding the various overlaps between growth and defense processes in *C. roseus* may lead to novel methods to overcome the growth versus defense compromise to ultimately increase yields of important pharmaceutical compounds.
6.2. Materials and Methods

6.2.1. C. roseus seedling growth

*Catharanthus roseus* seeds (cv. Vinca Little Bright Eye, NEseed) were sterilized and planted as previously described (Rizvi et al. 2014). In short, *C. roseus* seeds were surface-sterilized using 80 % (v/v) ethanol for 3 min followed by 30 % (v/v) bleach containing 0.1 % (v/v) Triton X-100 for 5 min. The seeds were rinsed 5 times and then soaked in sterile water for 24 h prior to being planted 1 mm below the surface of ~80 mL sterile solid full-strength Gamborg’s media (3.1 g/L Gamborg’s B-5 salts, 1 mL/L 1000X Gamborg’s vitamins, 5 g/L agar, pH = 5.7) in sterile Magenta GA-7 boxes. Seedlings were grown in the dark for one week and then transferred to a 16 h photoperiod.

6.2.2. Preparation of virus-induced gene silencing (VIGS) constructs

Small pieces of the protoporphyrin IX magnesium chelatase subunit H (*Chlh*) and *Zct1* were amplified from *C. roseus* cDNA using attB-containing primers (Table 6.16). *Chlh*, involved in chlorophyll biosynthesis, was previously used as a marker for VIGS silencing, and the same piece was used for silencing in this study (Liscombe and O’Connor 2011). A fragment of *Gfp* was amplified from the pPD95_77 vector (addgene.org plasmid #1495) to create a control for the effects of RNAi (Table 6.16). All three pieces were cloned separately into pDONR221 using Gateway Technology (Life Technologies). Constructs were confirmed by restriction digest (final destination vectors below were sequenced and therefore confirmed the accuracy of the pDONR construct sequence). pTRV1 and pTRV2-gateway were obtained from the Arabidopsis Biological Resource Center. The pDONR221- *Chlh*, -*Zct1*, and -*Gfp* constructs were digested with MluI and NruI to remove the kanamycin resistance and recombined into pTRV2-gateway using Gateway Technology. DH5α *Escherichia coli* competent cells were used for cloning (Z-
competent *E. coli* Transformation Kit). *E. coli* was grown in LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl with 15 g/L agar for plates, pH= 7.0) at 37°C overnight with shaking at 250 rpm when in liquid culture. Antibiotic resistance conferred by each vector and concentrations used for selection are listed in Table 6.16. The pTRV1 and pTRV2-Chlh, -Zct1, and -Gfp plasmids were electroporated into *Agrobacterium rhizogenes* R1000 (ATCC 43056) as previously described, except LB media was used instead of YM (Rizvi et al. 2014; Weaver et al. 2014). R1000 was grown at 26°C for 2-3 days with shaking 250 rpm when in liquid culture.

### Primers for pDONR221 constructs for VIGS

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Table 6.16: Primers used for cloning *CHLH*, *Zct1*, and *GFP* into VIGS vectors and antibiotic resistance conferred by each vector.

### 6.2.3. VIGS infections of *C. roseus* seedlings

Approximately six-week old *C. roseus* seedlings, that did not have the second set of true leaves, were transformed as previously described (Rizvi et al. 2014), except *A. rhizogenes* R1000 pTRV1 culture was mixed with either the pTRV2-Chlh, -Zct1, or -Gfp culture in a 1:1 ratio before infection. Infected seedlings were kept in the dark for 24 h before being maintained at a 16 h photoperiod. The first two leaf-pairs that emerged after infection were harvested in liquid
nitrogen when the CHLH controls presented a photo-bleached phenotype in these leaf-pairs (~6-7 weeks after transformation).

**6.2.4. Induction with GA, JA and 17β-estradiol**

*C. roseus* seedlings were elicited with GA, JA or both. Stock solutions of JA were prepared in ethanol (200 proof, Decon Laboratories), and added to 50 mL of Gamborg’s half-strength liquid media (1.55 g/L Gamborg’s B-5 salts, 1 mL/L 1000X Gamborg’s vitamins, pH = 5.7) in a sterile 250 mL flask for a final concentration of 1000µM JA. 1 mg/mL gibberellic acid solution (G198; Phytotech Laboratories) was also added to 50 mL Gamborg’s media for a final concentration of 100µM GA. 3-week old seedlings were placed in these liquid cultures for 0.5 and 8 h.

Similarly, *C. roseus* hairy root cultures were elicited with JA, GA or both. Stock solutions were prepared as above, and on day 21 (mid-exponential growth phase) hairy root cultures (50 mL) were elicited to reach a final concentration of 1000µM JA and/or 100µM GA. Cultures were harvested 0.5 and 8 h after treatment.

To study *Zct1*-silencing in *C. roseus* hairy roots, transgenic, estrogen-inducible *Zct1*-silenced hairy roots were used (line *Zct1hp*-38, generated previously) (Rizvi et al.). To induce *Zct1*-silencing, 17β-estradiol (Sigma Aldrich) stock was prepared in DMSO and added to hairy root cultures (50 mL) for a final concentration of 5µM.

**6.2.5. Gene expression analysis by qPCR**

mRNA was extracted from *C. roseus* seedlings using the RNAzol® RT (Molecular Research Center) method and quantified using a NanoDrop (ND-1000 Spectrophotometer; ThermoScientific). Extracted RNA was treated with DNase to remove genomic DNA, and cDNA was synthesized from the mRNA (1–5 µg; Deoxyribonuclease I Amplification Grade, SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen). Expression of *Chlh*, *Zct1*,
Zct2, Zct3, and Della (and Rps9 as the housekeeping gene) were monitored using primer designs previously described (Goklany et al. 2013; Liscombe and O'Connor 2011) (Table 6.17). Preparation for qPCR was performed using the RT² Real-Time SYBR Green/ROX PCR master mix (SABiosciences). qPCR was performed (QuantStudio 6, Applied Biosystems) using the thermocycler protocol previously described (Goklany et al. 2013). Fold changes were calculated using the ΔΔCt method.

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</table>

Table 6.17: Primers used to monitor gene expression through qPCR. * primers adapted from (Liscombe and O’Connor 2011)
6.3. Results and Discussion

6.3.1. Silencing Zct1 leads to growth defects in C. roseus seedlings

Zct1 expression was minimized using virus-induced gene silencing (VIGS), a transient silencing method previously used in C. roseus seedlings (Liscombe and O'Connor 2011). Zct expression significantly increases in the Gfp-silenced control (Zct1, p = 0.02; Zct2, p = 0.02), indicating that the VIGS method induces a defense response in C. roseus (Figure 6.35), similar to other transient transformation methods used (Weaver et al. 2014). While Zct2 and Zct3 are highly homologous to Zct1, only Zct1 expression in Zct1-silenced seedlings is significantly decreased compared to the Gfp-silenced control (p = 0.04).

Interestingly, seedlings with significant Zct1-silencing exhibit severely stunted growth (Figure 6.36). Neither the Gfp-silenced nor Chlh-silenced (Protoporphyrin IX magnesium chelatase subunit H, which is involved in chlorophyll biosynthesis) controls exhibit this growth defect (Figure 6.36 and Figure 6.37), indicating the growth defect is attributed to Zct1-silencing. Since neither Zct2 nor Zct3 are silenced in the Zct1-silenced samples, the observed growth defect is not associated or compensated by these zinc fingers. This is not surprising since ZCT3 is structurally different than ZCT1 and ZCT2, and has been shown to play both overlapping and differing roles in regulation (Chebbi et al. 2014; Pauw et al. 2004). To further confirm their differing role on growth, Zct2 and Zct3 were individually silenced (VIGS), and no growth defect is seen (data not shown).
Figure 6.35: Zct1, Zct2 and Zct3 expression in Zct1-silenced (VIGS) C. roseus seedlings. Expression levels were monitored in 4-5 leaf-pairs per condition. Rps9 was used as the housekeeping gene, and fold changes were calculated using the ΔΔCt method relative to one untreated seedling (denoted with a fold change of 1.0). Bars indicate the mean and standard deviations for each condition. Significance was calculated using the Student’s t-test (* denotes $p < 0.05$).
Figure 6.36: Growth of untreated, Gfp-silenced, and Zct1-silenced C. roseus seedlings.

Figure 6.37: CHLH expression in CHLH-silenced (VIGS) C. roseus seedlings. CHLH expression was monitored in 2-4 leaf-pairs per condition. Rps9 was used as the housekeeping gene, and fold changes were calculated using the ΔΔCt method relative to one untreated seedling (denoted with a fold change of 1.0). Bars indicate the mean and standard deviations for each condition. Significance was calculated using the Student’s t-test (* denotes p < 0.05).
To check if Zct1 is similarly involved in growth of *C. roseus* hairy roots, stably transformed hairy roots with inducible Zct1-silencing were studied (Rizvi et al. In submission). Zct1-silencing was induced in these hairy root cultures with 5µM 17β-estradiol on day 15 of growth cycle and compared to untreated cultures. Growth in the Zct1-silenced line was reduced, especially after day 30, but not significantly lower than the untreated controls (Figure 6.38). Although Zct1 levels are significantly lower upon 17β-estradiol treatment in this line (Rizvi et al. In submission), there is no observed growth defect in *C. roseus* hairy roots as seen in *C. roseus* seedlings.

![Graph](image)

**Figure 6.38**: Fresh weight of Zct1-silenced *C. roseus* hairy roots (Zct1hp-38). 5µM 17β-estradiol was added to cultures on day 15 of growth to induce Zct1-silencing, and the fresh weight (g/L culture) was recorded every 5 days for estradiol treated and untreated cultures. Data points are averages of 5 biological replicates and error bars denote standard deviations.

The difference in growth inhibition upon silencing Zct1 in *C. roseus* seedlings but not hairy roots is likely due to the differential control in growth between hypocotyls and roots. DELLA is the master regulator of growth inhibition between both tissue types. For example, DELLA proteins interact with PIFs (bHLH proteins) to inhibit hypocotyl elongation (Sun 2011). DELLA also binds to the SCARECROW-LIKE 3 (SCL3) protein, which plays a role in root
development (Ubeda-Tomás et al. 2008). Our results suggest that ZCT1 may be associated not only with a defense-response, but also with shoot-specific growth potentially through DELLA regulators.

6.3.2. Zct1 promoter contains both JA- and GA-responsive elements

Since Zct1 is involved in both defense and growth in C. roseus seedlings, approximately 2kb of the Zct1 promoter was sequenced and regulatory elements were identified using the Plant Cis-Acting Regulatory Elements (PlantCARE) database (Weaver et al. In submission).

Interestingly, both JA- and GA-responsive elements were found on the Zct1 promoter, located in two distinct, clusters. Two activation sequence 1 (AS-1) type JA-responsive elements are located between -108 and -138 relative to the transcriptional start site (TSS) on the Zct1 promoter (Figure 6.39). AS-1 elements are essential for JA inducibility in the Agrobacterium T-DNA nopaline synthase (nos) promoter in tobacco, the barley lipoxygenase 1 (lox1) promoter, the Cauliflower Mosaic Virus (CaMV) 35S promoter, and the C. roseus Dat and Wrky1 promoters (Memelink 2009; Wang et al. 2010b; Yang et al. 2013).

In addition to JA-responsive elements, several GA-responsive elements were identified (Figure 6.39) (Weaver et al.). GA-responsive element (GARE)-motifs are typically bound by GA-responsive transcription factors and activate transcription of GA-responsive genes. Many promoters of DELLA-regulated genes contain GARE-motifs (Stamm et al. 2012), indicating that Zct1 may in fact be regulated by DELLA through an intermediate transcription factor. Two binding sites for IDD proteins (AGACAA) were also identified on the Zct1 promoter. IDD are C2H2 zinc finger proteins that interact with both DELLA proteins and GA-responsive gene promoters in Arabidopsis (Feurtado et al. 2011; Yoshida et al. 2014). The presence of this cluster of 6 putative GA-responsive elements in the Zct1 promoter is consistent with its role in growth.
6.3.3. Zct expression is modulated by GA treatment in C. roseus seedlings

Since the Zct1 promoter contains a cluster of JA- and GA-responsive elements, we experimentally explored the responsiveness of Zct1 expression to JA and GA. In Arabidopsis, GA affects the expression of JA-responsive genes through DELLAs (Hou et al. 2010). To test if GA is similarly involved in JA-induced Zct expression in C. roseus, the expression of Zct1, Zct2, and Zct3 was monitored under JA and/or GA treatment. C. roseus seedlings were treated with 100µM GA, 1000µM JA, or the combination for 0, 0.5, and 8 h. This JA dosage is known to induce Zct expression (Goklany et al. 2013), and a dosage experiment was done to determine the most effective GA dosage (data not shown).

Expression of Zct1, Zct2, and Zct3 increases significantly in seedlings treated with 1000µM JA (Figure 6.40), as previously reported in hairy roots (Goklany et al. 2013). Although GA treatment alone minimally induces Zct expression, the combined treatment of GA and JA significantly reduces the induction by JA (Figure 6.40). These results suggest that GA suppresses
cellular response to JA, similar to that observed in *Arabidopsis* (Hou et al. 2010). *C. roseus* hairy roots were also treated with 100µM GA, 1000µM JA, or the both for 0, 0.5, and 8 h. Unlike seedlings, *Zct1* induction by JA in hairy roots is not suppressed by GA (Figure 6.41). As with *Zct1*-silencing, hairy roots and seedlings respond differently to the combined treatment of GA and JA.

In the proposed “relief of repression” model, DELLAs compete with MYC2 for binding to JAZ in *Arabidopsis* (Hou et al. 2010; Yang et al. 2012). In the absence of GA, DELLA proteins are stabilized and bind to JAZ, releasing MYC2 to promote JA-signaling. In the presence of GA, however, DELLA proteins are degraded, which releases JAZ to bind and repress MYC2, thus attenuating JA-signaling (Hou et al. 2010; Yang et al. 2012). The attenuation of JA-induced *Zct* expression by GA suggests that DELLA proteins are also involved, either directly or indirectly, in modulating *Zct* expression in *C. roseus* seedlings.
Figure 6.40: Zct1, Zct2 and Zct3 expression in *C. roseus* seedlings treated with JA and/or GA. Gene expression was normalized to *Rps9* and fold changes were calculated relative to the untreated (0 h) time point. Data points are averages of 2 seedling replicates and error bars denote standard deviations.

Figure 6.41: Zct1 expression in *C. roseus* hairy roots treated with JA and/or GA. Gene expression was normalized to *Rps9* and fold changes were calculated relative to the untreated (0 h) time point. Data points are averages of 2 biological replicates and error bars denote standard deviations.
6.3.4. Identification and analysis of DELLA in *C. roseus*

GA suppresses JA-induced *Zct1* expression in *C. roseus* seedlings, which is potentially mediated through DELLA. Although the DELLA family of transcription factors is highly conserved in plants, they have not been identified in *C. roseus*. There are five DELLA proteins in *Arabidopsis*: RGA, GAI, RGL1, RGL2 and RGL3 (Sun 2011). Using the Medicinal Plant Genomics Resource (http://medicinalplantgenomics.msu.edu/), we identified one *Della* transcript in *C. roseus* that is homologous with all five *Arabidopsis* DELLA genes (Table 6.18). Similarly, rice and barley each have a single DELLA protein (Chandler et al. 2002; Fu et al. 2002; Ikeda et al. 2001).

<table>
<thead>
<tr>
<th><em>Arabidopsis</em> DELLA proteins</th>
<th><em>C. roseus</em> transcriptome BLAST</th>
<th><em>Arabidopsis</em> and <em>C. roseus</em> protein similarities:</th>
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</thead>
<tbody>
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<td>Locus ID:</td>
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<td>RGL1</td>
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<tr>
<td>RGL3</td>
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Table 6.18: BLAST results for five *Arabidopsis* DELLA gene sequences (RGA, GAI, RGL1, RGL2 and RGL3) matching one *C. roseus* transcript ID from the Medicinal Plant Genomic Resource database (http://medicinalplantgenomics.msu.edu/).
Della expression was monitored in C. roseus seedlings treated with 100µM GA. Della expression increased significantly and rapidly within 0.5 h, and stayed elevated even after 8 h (Figure 6.42). Since DELLA proteins are rapidly (< 0.5 h) degraded upon GA-treatment (Zentella et al. 2007), rapid induction of Della transcripts may be required in order to re-repress DELLA targets, similar to the rapid induction of Jaz transcripts with JA treatment in Arabidopsis and C. roseus (Rizvi et al. 2013; Thines et al. 2007; Zhang 2008). Upon JA treatment, JAZ is degraded, releasing MYC2 proteins to induce Jaz expression and leading to the re-repression of MYC2 (Chini et al. 2007).

C. roseus hairy roots were also treated with 10 or 100µM GA for 0, 0.5, and 8 h. Surprisingly, Della transcripts did not significantly increase with GA treatment in hairy roots (Figure 6.43). C. roseus hairy roots are generated by an agropine-type Ri plasmid (R1000) which contains four rol genes (rolA, rolB, rolC and rolD) and genes representing two steps of auxin biosynthesis (aux1 and aux2, or also known as tms1 and tms2) thought to be responsible for the formation of hairy roots (Nemoto et al. 2009). This altered hormone level and metabolism (through the Agrobacterium-mediated transformation) may be responsible for the lack of GA-response in C. roseus hairy roots. Furthermore, since C. roseus hairy roots exhibit fast growth, they may already have a high level of native GA levels. The difference in the responsiveness of Della expression with GA between hairy roots and seedlings is consistent with the previous sections.
Figure 6.42: *Della* expression in *C. roseus* seedlings treated with 100µM GA. Gene expression was normalized to *Rps9* and fold changes were calculated relative to the untreated (0 h) time point. Data points are averages of 2 seedling replicates and error bars denote standard deviations.

Figure 6.43: *Della* expression in *C. roseus* hairy roots treated with 10µM or 100µM GA. Gene expression was normalized to *Rps9* and fold changes were calculated relative to the untreated (0 h) time point. Data points are averages of 2 biological replicates and error bars denote standard deviations.
In line with our hypothesis that DELLA functions as a master regulator between growth and defense in *C. roseus*, we will test the interaction of DELLA with growth-related (PIF) and defense-related (JAZ) proteins. *C. roseus* has two JAZ proteins, CrJAZ1 and CrJAZ2 (Table 6.19). CrJAZ1 has been shown to repress *C. roseus* MYC2 and is more extensively studied (Zhang 2008), therefore CrJAZ1 will be tested for binding to DELLA in *C. roseus*. *Arabidopsis* contains two PIF proteins that are involved in promoting growth process and bind to DELLA, PIF3 and PIF4 (Sun 2011). These *Arabidopsis* gene sequences were used to identify homologous PIF proteins in *C. roseus* using the Medicinal Plant Genomics Resource (Table 6.19). CrPIF4 protein is homologous to both AtPIF3 and AtPIF4, so CrPIF4 will be tested for binding to DELLA in a Y2H screen (in progress). CrDELLA, CrPIF4 and CrJAZ1 are being amplified from cDNA, cloned into the pDONR Gateway vector, and then recombined into the Y2H vectors. The CrDELLA-CrPIF4 and CrDELLA-CrJAZ1 interactions will be tested for reporter gene activation. Importantly, several controls will be done including vector-only controls, weak, moderate, and strong interactors, and expression of GAL4 itself. This is important considering DELLA proteins have strong self-transactivation activity (Yoshida et al. 2014).

<table>
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<tr>
<th>Protein name:</th>
<th><em>Arabidopsis</em> proteins responsible for:</th>
<th>NCBI GenBank ID:</th>
<th><em>C. roseus</em> transcriptome BLAST Locus ID:</th>
<th><em>C. roseus</em> protein name:</th>
<th>Similarity to <em>Arabidopsis</em> proteins:</th>
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Table 6.19: BLAST results for five *Arabidopsis* JAZ and PIF gene sequences and the homologous *C. roseus* transcript IDs from the Medicinal Plant Genomic Resource database (http://medicinalplantgenomics.msu.edu/). *C. roseus* proteins used for a Y2H assay are highlighted in red.
6.3.5. Arabidopsis IDD proteins are highly homologous to C. roseus IDD proteins

We hypothesize that DELLA may be involved in regulating Zct and its role in growth and defense through a class of INDETERMINATE Domain (IDD) proteins. This recently discovered protein class (Feurtado et al. 2011; Yoshida et al. 2014) serves as a potential intermediate between DELLA and downstream genes such as Zct in C. roseus. When IDD was repressed (fused with SRDX, a dominant repressor domain (Hiratsu et al. 2003)), severe growth defects, including smaller leaves and shorter roots, were observed in Arabidopsis (Yoshida et al. 2014), similar to the effects seen when Zct1 was silenced in C. roseus seedlings (Figure 6.36). Since the Zct1 promoter contains putative IDD binding motifs (Figure 6.39), IDD may be involved upstream of ZCT1 by regulating its expression.

We identified three IDD proteins in C. roseus that are homologous to the six IDD proteins in Arabidopsis (Table 6.20) (Feurtado et al. 2011; Yoshida et al. 2014). The alignment of these six Arabidopsis IDD proteins with the three C. roseus IDD proteins shows conserved zinc finger domains, as well as a highly conserved MSATALLQKAA and TR/LDFLG domain (Figure 6.44). Either the MSATALLQKAA and TR/LDFLG domains or just the TR/LDFLG domain is needed for IDD proteins to bind to DELLA (Yoshida et al. 2014).

<table>
<thead>
<tr>
<th>Arabidopsis IDD proteins</th>
<th>C. roseus transcriptome BLAST</th>
<th>C. roseus protein name:</th>
<th>Similarity to Arabidopsis proteins:</th>
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<td>Locus ID:</td>
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Table 6.20: BLAST results for six Arabidopsis IDD gene sequences matching three C. roseus transcript IDs from the Medicinal Plant Genomic Resource database (http://medicinalplantgenomics.msu.edu/). * incomplete coding sequence, so this IDD protein was not analyzed.
Figure 6.44: Protein alignments of six Arabidopsis IDD proteins (AtIDD1, AtIDD3, AtIDD9, AtIDD10, AtIDD4, and AtIDD5) with two *C. roseus* IDD proteins (CrIDD1 and CrIDD2). The Arabidopsis and *C. roseus* IDD proteins contain the highly homologous zinc finger domain, MSATALLQKAA domain, and TR/LDFLG domain.
6.4. Future Directions

6.4.1. Test the binding of the DELLA protein to IDD proteins in *C. roseus*

To test the interaction between CrIDD and CrDELLA proteins, a Y2H assay (Invitrogen Gateway) will be performed. If these proteins have similar function to *Arabidopsis*, both CrIDD1 and CrIDD2 should bind to the CrDELLA protein. *C. roseus* DELLA and IDD will be amplified from cDNA, cloned into the pDONR Gateway vector, and then recombined into the Y2H vectors. The CrDELLA-CrIDD interaction will be subjected to four tests for reporter gene activation (Growth on –His media, the β-galactosidase assay, and two independent tests for URA3 activation); this quadruple check for interactions reduces false positives. Each assay includes a panel of controls, including vector-only controls, weak, moderate, and strong interactors, and expression of GAL4 itself. This is important considering DELLA proteins have strong self-transactivation activity (Yoshida et al. 2014).

6.4.2. Test the binding of *C. roseus* IDD proteins bind to the Zct1-promoter

The Zct1-promoter contains two IDD-binding domains: AGACAA (Figure 6.39) (Weaver et al. In submission). If the CrDELLA protein binds to the CrIDD proteins, we will then test if the CrIDD proteins can also bind to the Zct1 promoter. Direct binding of the two IDD candidates, CrIDD1 and CrIDD2, to the Zct1 promoter will be analyzed by an electrophoretic mobility shift assay (EMSA). A Zct1 promoter fragment containing the two IDD-binding sites and fragment with mutated IDD-binding sites will be purchased and 3’ biotin-labeled. The two CrIDDs will be cloned into a vector for N-terminal His-tagged protein expression. The tagged proteins will be expressed in *E. coli* BL21-A1 cells and purified using His Spintrap Columns. A chemiluminescent EMSA will be performed to determine if the two CrIDDs can bind to the Zct1 promoter fragments containing the wild type and mutant IDD-binding sites. We expect the IDDs
will bind and shift the Zctl promoter fragment containing the IDD-binding sites, but not the mutant IDD-binding sites. These results are important because they illustrate a direct binding potential of the CrIDD proteins to the Zctl promoter.

6.4.3. Investigate the transactivation of Zctl by the DELLA-IDD complex

If the CrIDD proteins bind to the Zctl promoter, we will also verify that the DELLA-IDD complex can transactivate the Zctl promoter. Using particle bombardment and excised young leaves, two plasmids, the Zctl promoter driving Gus expression and an overexpression construct containing candidate IDDs or GFP (control), will be co-transformed with and without an overexpression construct for DELLA. We will use particle bombardment since Zctl is strongly induced in transient Agrobacterium transformation methods (Weaver et al. 2014).

Transactivation strength will be monitored using the MUG assay for Gus expression and normalized for particle bombardment transformation efficiency between samples using a Luciferase assay. The resulting values of the transactivation of the candidate IDDs +/- DELLA will be compared to the negative control (GFP) to determine if those proteins increased or decreased expression of the Gus reporter gene. We expect an increase in the expression of the Gus reporter gene driven by the Zctl promoter when overexpressing candidate IDDs and DELLA, but not IDDs alone. This will confirm IDD’s ability to act as an intermediate for DELLA in the regulation of Zctl expression in planta.
6.5. Conclusion

In this study, we have shown that ZCT1 regulates growth in *C. roseus* seedlings and that JA-induced *Zct1* expression in seedlings is modulated by GA, suggesting the involvement of DELLA in regulating *Zct1* expression. This response is associated with the seedlings as neither *Zct1* nor *Della* expression is responsive to GA in the hairy roots.

As the DELLA-PIF and DELLA-JAZ cascade in *Arabidopsis* has not been demonstrated in *C. roseus*, we will confirm that the CrDELLA protein directly binds to CrJAZ1, a defense-related protein, as well as CrPIF4, a growth-related protein. DELLA’s promiscuity in binding partners lends one point of regulation between growth and defense. CrDELLA may also bind to the newly characterized CrIDD proteins. We will demonstrated that these CrIDD proteins bind to the putative binding motifs in the *Zct1* promoter. Thus showing ZCT1 is regulated by CrDELLA proteins through CrIDD proteins, which will provide yet another point of modulation between the defense- and growth- signaling pathways. The decrease in JA-induced *Zct1* expression by GA is consistent with this model. While JA increases *Zct1* expression (through ORCA3 and other unknown cascades), GA decreases *Zct1* expression in two ways: 1) the GA-induced degradation of CrDELLA releases CrJAZ proteins to repress CrMYC2 and *Orca* expression, and thus decreasing *Zct1*, and 2) GA-induced degradation of CrDELLA obliterates its interaction with CrIDD necessary in activating *Zct1* expression. In the presence of both JA and GA, these competing signaling cascades dampen the induction of *Zct1* by JA. In conclusion, we will demonstrate ZCT1 is involved in both growth and defense, and that this involvement is mediated through DELLA and IDD proteins in *C. roseus* (Figure 6.45).
Figure 6.45: Protein and gene interactions involved in alkaloid biosynthesis and growth in *C. roseus*. Dashed lines indicate indirect or unknown interactions. * shown in (Weaver et al. In submission).
6.6. Author contributions

NFR conducted the *C. roseus* seedling and hairy root experiments (including hormone dosages and qPCR), bioinformatics using the *C. roseus* transcriptome, and wrote the initial version of this manuscript. JW cloned the VIGS constructs and generated the *A. rhizogenes* with each construct, conducted promoter experiments, and performed yeast-2-hybrid experiments. JW, EJC, and CLP were all involved in troubleshooting experiments and revising the manuscript. All authors read and approved the manuscript.

6.7. Acknowledgements

This work was supported by National Science Foundation (NSF) CBET Award #1033889 to CLP and EJC and by the American Association of University Women (AAUW) Dissertation Fellowship to NFR.
7.0 Conclusions and Recommendations

The overall goal of this research was to enhance the production of important pharmaceuticals from plant cell cultures to make them cost-effective and available for the treatment of patients. Specifically, we were interested in increasing the production of anticancer compounds from hairy root cultures of *C. roseus*. In this thesis, we first optimized an efficient *Agrobacterium*-mediated method for the genetic transformation of *C. roseus* hairy root cultures (33% transformation efficiency), including a novel, estrogen-inducible construct for transgene expression (Chapter 4). Unlike previous constructs, this inducible system did not elicit a defense response in *C. roseus* hairy roots and therefore is ideal for studying TIA production and regulation. This optimized transformation method and inducible construct was then used to create transgenic *C. roseus* hairy roots with silenced Zct1 expression (Chapter 5). While levels of Zct1 were significantly silenced, even in the presence of the elicitor MJ, TIA production did not increase further than compared to elicitation with MJ alone. Gene profiling indicated that the lack of increase in TIA levels was strongly correlated with TIA biosynthetic gene expression, which also did not increase further with Zct1-silencing. Expression of Orca2 and Orca3 was strongly correlated with TIA gene expression and TIA production. Interestingly, due to high homology, levels of Zct2 were also silenced in Zct1-silenced lines, even under MJ-elicited conditions.

- **Recommendation 1: Silence Zct1, Zct2 and Zct3 simultaneously**

  Although Zct1 and Zct2 were silenced, TIA production was low. Levels of Zct3, however, remained high. The three homologous ZCT repressors are thought to play overlapping but distinct functions in *C. roseus*. Therefore, silencing the expression of all three ZCT repressors simultaneously may inhibit other ZCTs from repressing TIA genes and...
potentially result in increased TIA production. *C. roseus* hairy root cultures transformed with the estrogen-inducible silencing of *Zct*1, *Zct*2 and *Zct*3 should therefore be generated, tested for TIA production, and compared to these results of silencing *Zct*1 alone.

**Recommendation 2: Investigate the dosage dependence and regulation of ORCA**

TIA production and TIA biosynthetic gene expression in *Zct*1-silenced hairy roots were still MJ-dosage dependent (high at 250µM MJ and low at 1000µM MJ). While the MJ-induction and dosage dependence of *Zct*1 was abolished in *Zct*1-silenced lines, levels of ORCA activators were still strongly correlated with MJ-dosage-dependent TIA production. Although previous attempts to increase ORCA levels did not significantly increased TIA levels (Pan et al. 2012; Peebles et al. 2009), the correlation of ORCA with MJ-induced TIA production indicates the importance of ORCA in regulation of TIAs. Understanding what regulates and is causing the MJ-dosage dependence of ORCA may unveil stronger targets for metabolic engineering.

In addition to silencing *Zct*1 in hairy roots, *Zct*1 expression was also silenced in *C. roseus* seedlings through virus-induced gene silencing (VIGS; Chapter 6). Surprisingly, ZCT1 was involved in regulating growth in seedlings (but not hairy roots). Not only does the *Zct*1 promoter contain both JA- and gibberellin (GA)- elements, but JA-induced *Zct*1 expression in seedlings was also modulated by GA, further suggesting that ZCT1 plays a role in both defense and growth. This phenomenon is similar to the involvement of *Arabidopsis* DELLA proteins, which regulate both growth (by binding and regulating PIF proteins) and defense (by binding and regulating JAZ proteins). Using the *C. roseus* transcriptome, one DELLA protein (CrDELLA) was identified and is GA-responsive.
• **Recommendation 3: Verify DELLA proteins modulate both growth and defense in**

*C. roseus*

As the DELLA-PIF and DELLA-JAZ cascade in *Arabidopsis* has not been demonstrated in *C. roseus*, the binding of CrDELLA protein directly to CrJAZ1, a defense-related protein, as well as CrPIF4, a growth-related protein, should be verified *in vitro* through a Y2H assay. This would confirm that DELLA proteins bind to proteins in both GA- and JA-signaling cascades in *C. roseus*, similar to *Arabidopsis*.

We also identified three IDD proteins in *C. roseus*, which are highly homologous to the *Arabidopsis* IDD proteins. IDD proteins in *Arabidopsis* bind to and serve as an intermediate between DELLA proteins and DELLA-regulated genes. Analysis of the *Zct1* promoter revealed two IDD-binding sites (AGACAA).

• **Recommendation 4: Investigate CrIDD proteins as an intermediate between DELLA and Zct1**

Because IDD proteins bind in DELLA in *Arabidopsis*, the interaction between CrIDD (two proteins identified) and CrDELLA should be verified *in vitro* by a Y2H assay. If these proteins have similar function to *Arabidopsis*, both CrIDD1 and CrIDD2 should bind to the CrDELLA protein. If the CrDELLA protein binds to the CrIDD proteins, the interaction between CrIDD proteins and the *Zct1* promoter should also be tested *in vitro* through a Y1H or an EMSA. Since the role of *Zct1* as a repressor of TIA genes is already known, this study would elucidate specifically how *Zct1* is involved in growth and GA-response. If the CrIDD proteins bind to the *Zct1* promoter, the transactivation of *Zct1* by the DELLA-IDD complex should also be tested by particle bombardment. This will confirm the ability of CrIDD to act as an intermediate for DELLA in the regulation of
*Zct1* expression *in planta*. Through these studies, it can be shown that *Zct1* is regulated by CrDELLA proteins through CrIDD proteins, which, in addition to DELLA, will provide yet another point of modulation between the defense- and growth- signaling pathways.

Overall, this research adds to the understanding of how important plant products are regulated and strategies to increasing their production. The combination of elicitors and metabolic engineering of key transcription factors is a promising strategy for increasing production. By further elucidating the transcriptional network, with help from the recently established *C. roseus* genome, and pursuing other/additional transcription factor targets, it may soon be economically feasible to produce of TIAs from *C. roseus* hairy root cultures in large-scale bioreactors. Furthermore, the tradeoff between growth and defense in plants has been well documented for decades but poorly understood. Uncovering the mechanism by which plants switch between growth and defense processes may lead to novel methods to overcome this compromise to ultimately increase yields and reduce costs of important pharmaceutical compounds.
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