MECHAMISM OF JASMONIC ACID REGULATION OF TERPENOID INDOLE ALKALOIDS IN THE LEAVES OF CATHARANTHUS ROSEUS

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE CHEMICAL ENGINEERING

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JUNE 2019

Acknowledgments

I would like to thank my advisor Dr. Guy Sander for teaching me the principles of biochemical engineering, providing great consultation and supporting me throughout the duration of this work.

I would also like to thank the faculty of the Chemical Engineering department at the University of Minnesota Duluth for teaching me the principles of chemical engineering through academic coursework.

Of these faculty members, I would like to specifically thank Professor Richard Davis and Dr. Victor Lai for serving on my thesis committee.

I would also like to thank my friends and fellow graduate students in the Chemical Engineering department for their support and relationships both on- and off-campus.

Finally, I'd like to thank my family for supporting me throughout the entirety of my professional education. I would like to dedicate this work to my father, Matthew Lee Iwen.

Funding for this project was provided by the Chancellor's Faculty Small Grant and Teaching & Research assistantships through the University of Minnesota Duluth.

Abstract

Catharanthus roseus produces terpenoid indole alkaloids (TIAs) including the pharmaceutically significant vincristine and vinblastine. Vincristine and vinblastine are two complex dimeric compounds, derived by the coupling of vindoline and catharanthine. The yields of vincristine and vinblastine from C. roseus are remarkably low due to their cytotoxicity and the localized production of their monomeric compounds. Jasmonate hormones have been shown to activate transcription factors known to regulate several biosynthetic genes in the TIA pathway. Leaves of mature C. roseus plants were sprayed with Jasmonic Acid and harvested from zero to 96 hours after administration, homogenized, and incubated in a buffer solution. Leaves were also sprayed with clotrimazole and harvested from zero to four hours and alkaloids extracted in Methanol. Results showed no formation of vincristine and vinblastine and high variability in measured alkaloid levels. A complex mechanism of JA acting between neighboring leaves of C. roseus is supported by these results.

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Background

Introduction

Terpenoid Indole Alkaloids (TIAs) are a class of biological products containing a tryptophan or tryptamine component. Some TIAs are thought to function as a defense mechanism against fungal infection and insect folivory [1]. The mechanism of defense varies depending on the alkaloid. For example, strictosidine can be deglucosylated to form a highly reactive aglycone, which causes protein reticulation; while vincristine and vinblastine disrupt microtubule formation during mitosis, causing dissolution of spindles and hindering the division of replicating cells [1], [2]. The defense mechanisms of Vincristine and Vinblastine led to them being the first biologically produced anti-cancer drugs.

Vincristine and vinblastine are two highly complex dimer TIAs produced via the coupling of vindoline and catharanthine, two monoterpeniods made in the monoterpeniod pathway of C. roseus [3]. Using modern isolation techniques, it requires 500kg of dried C. roseus leaves to obtain only 1g of vinblastine [4]. This has prompted extensive research into increasing the production of these metabolites. Alternative approaches to their production, such as chemical synthesis and production in micro-organisms, were unsuccessful due to the complex structure and biological synthesis of these compounds [5].

One of the factors contributing to the low yields of dimer TIAs in C. roseus is their spatial separation within the plant [6], [7]. The pathway producing catharanthine and its precursors has been found to be compartmentalized in multiple cell types in the above-

ground organs including the leaves, stems, and flowers of the plant. The first step in this pathway has been localized to specialized internal phloem parenchyma (IPAP) cells [8] and the accumulation of catharanthine has been further localized to the leaf surfaces where it also facilitates antifungal properties [7]. Whereas vindoline production and accumulation has been found in internal laticifer and idioblast cells [6]. This isolation is beneficial to the plant because the anti-mitotic defense mechanism is only utilized when folivorous attack facilitates the breaking of all cells within the leaf, allowing for the combination of the monomers. When caterpillars were fed a diet of C. roseus, the alkaloid levels found in their gut had dimer to monomer ratios (anhydrovinblastine/catharanthine & vindoline) far higher than those found in the whole leaf [2].

Terpenoid Indole Alkaloid Biosynthesis

Figure 1and Figure 2show the steps of the terpenoid indole alkaloid pathway ending at vincristine; not shown are additional known branches off of tabersonine. The first terpenoid indole alkaloid in this pathway is strictosidine, shown in Figure 1. Strictosidine is formed by the condensation of tryptamine and secologanin by strictosidine synthase (STR) [5]. The terminal steps of secologanin production have been found to take place exclusively in the epidermal cells of the leaves via a membrane-associated cytochrome P450 (CYP450) mono-oxygenase, secologanin synthase (SLS) [9]. Strictosidine is deglucosylated by Strictosidine β-D-glucosidase (SGD) to form strictosidine aglycone, a protein reticulating compound [1].

Strictosidine aglycone is spontaneously dehydroxylated to form 4,21-dehydrogeissoschizine, which can be further derived to terminal products in three

separate pathways. The first of these pathways produces serpentine and ajmalicine. Other pathways from 4,21-dehydrogeissoschizine lead to the production of the important metabolites catharanthine and tabersonine [5]. Catharanthine and Tabersonine share a common precursor, however, Catharanthine is secreted to the epidermal cell surface while tabersonine accumulates within the epidermal cells [7].

Tabersonine is a node in the TIA pathway and the downstream conjugation is largely dependent on the location within the plant. The pathway in the roots produces eight monoterpeniod alkaloids of which include lochnericine, hörhammericine, and the terminal metabolites 19-acetoxyhörhammericine, and echitovenine. The leaf pathway consists of six enzymatic steps to produce the terminal monoterpeniod, vindoline. The leaf pathway is diagramed in Figure 2

The conversion of tabersonine to vindoline is facilitated by six enzymatic steps of which five have been characterized. While still in the epidermal cells, tabersonine is hydroxylated at the C-16 position by tabersonine-16-hydroxylase, an endoplasmic reticulum CYP450 mono-oxygenase [10], [11]. There has been accumulating evidence showing that tabersonine-16-hydroxylase activity is directly correlated to vindoline content, indicating that this step plays a key role in vindoline biosynthesis and accumulation [11], [12]. The second and third steps consist of O-methylation via 16-hydroxytabersonine-16-O-methyltransferase followed by subsequent hydroxylation via an uncharacterized enzyme, which moves the substrate from the epidermal cells to mesophyll cells and yields 10-methoxy-2,3-dihydro-3-hydroxytabersoine [6]. This compound then transported from the mesophyll cells to idioblast or laticifer cells by a chloroplast thylakoid-associated 2,3-dihydro-16-methoxytabersonine N-

methyltransferase (NMT) reaction and produces desacetoxyvindoline [7]. Desacetoxyvindoline-4-hydroxylase, a light dependent hydroxylase [13], and deacetylvindoline 4-O-acetyltransferase [14] catalyze the last two steps in vindoline biosynthesis pathway. Vindoline and catharanthine are then condensed by peroxidase 1 (PRX1) to make 3-4-anhydrovinblastine which is then oxidized further by uncharacterized enzymes to yield vincristine and vinblastine [5], [15].

Metabolic Engineering of TIA Metabolism

Due to the high toxicity of some TIAs, the pathways producing them are highly complex and regulated. The pathways producing TIAs involve over 35 intermediates, 30 biosynthetic genes, eight regulatory genes, numerous subcellular compartments, and at least 5 cell types [3], [6], [16]. Much effort has gone into identifying relationships between substrates and enzymes throughout the pathways in an effort to increase the production of downstream metabolites. Tryptamine is produced by the shikimate pathway and is later combined with secologanin to make strictosidine. Anthranilate synthase is one of the enzymes involved in the shikimate pathway and undergoes feedback inhibition by both tryptophan and tryptamine [17]. Feedback-insensitive anthranilate synthase α and α, β subunits have been successfully expressed in C. roseus hairy roots. This resulted in a 100-fold increase in tryptophan and two-fold increase in tryptamine and lochnericine levels; however, there was no evidence of accumulation of other downstream metabolites suggesting that the bottlenecks of the flux through the TIA pathway occur downstream of tryptophan, or that the supply of secologanin is limited [18]. Combining feedback-insensitive anthranilate synthase with the feeding of the terpenoid precursor 1-deoxy-D-xylulose to hairy root cultures showed significant increases in hörhammercine levels.

Alternatively, feeding feedback-insensitive anthranilate synthase with the terpenoid precursor loganin in hairy roots showed significant increases in catharanthine [19]. Overexpression of 1-deoxy-D-xylulose synthase, an enzyme thought to have high regulation in the production of secologanin, caused a significant increase in ajmalicine and lochnericine, and a significant decrease in tabersonine and hörhammericine in hairy root cultures. Co-expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase showed significant increases in ajmalicine, tabersonine, lochnericine, and total TIAs [20]. These studies show that precursor feeding and the manipulation of single enzymes to facilitate flux through a pathway has varying results, and that even the manipulation of multiple enzymes can be ineffective at producing the metabolite of interest.

Attempts have been made to produce TIAs in a heterologous host, Saccharomyces cerevisiae, to further identify the multicellular dependence of TIA production [21], [22]. Additionally, the dependence of microsomal plant P450 enzymes in TIA production can be better demonstrated in S. cerevisiae due to their higher precedence than in plants [23]. Strains of S. cerevisiae harboring 15 plant-derived, and five yeast derived overexpressed genes produced extracellular strictosidine fractions of ~0.5mg/L [21]. S. cerevisiae harboring genes necessary for the conversion of tabersonine to vindoline were able to produce 0.6 mg $gdwt^{-1}$ over 12 hours [22], in comparison to 2 mg $gdwt^{-1}$ after 50 days in seedlings [24].

Leaf cell suspension cultures have shown the ability to produce catharanthine and tabersonine, however they lack the necessary enzymes to produce vindoline from tabersonine [25]. Transgenic leaf cell suspension cultures have shown decreasing TIA

production and genetic stability over time [26]. The unreliability of transgenic leaf cell suspension cultures to survive and produce TIAs has shifted the focus of transgenic cell suspension cultures to hairy root cell cultures. The use of hairy root cultures has provided much knowledge into the effects of gene overexpression and mutation in C. roseus [18]–[20]. Furthermore, hairy root cultures show increased genetic stability compared to leaf cell suspension cultures [27]. The major bottleneck of hairy root cultures is their inability to produce vindoline from tabersonine due to their lack of laticifer and idioblast cells [28]. Attempts to increase the flux of the pathway leading to vindoline by overexpressing tabersonine-16-hydroxylase and 16-hydroxytabersonine-16-O-methyltransferase yielded changes in TIA gene transcription, and increases of metabolites not found in the vindoline biosynthesis pathway [29]. While heterologous hosts and hairy root suspension cultures are a useful way to characterize steps in the TIA biosynthesis pathway, they have yet to show the ability to produce vincristine and vinblastine.

Transcriptional regulators usually control the expression of multiple genes in a biosynthetic pathway. It can be speculated that controlling transcriptional regulators can enhance the production of metabolites of interest [5]. For example, the plant growth hormone auxin has been shown to downregulate the transcription of the tryptophan decarboxylase gene in C. roseus which is responsible for producing tryptamine, a precursor to the TIA pathway [30]. In the C. roseus TIA pathway, several transcription factors have been identified; among these transcription factors are Octadecanoid-Responsive Catharanthus AP2/ERF-domain (ORCA) 2 [31], ORCA3 [32], the C. roseus box P-binding factor (CrBPF) 1 [33], Catharanthus roseus E-box binding factors CrMYC1 [34] and CrMYC2 [35], Catharanthus roseus W-box binding factors

CrWRKY1 [36] and CrWRKY2 [37], and three Cys_2/His_2-type zinc finger proteins (ZCT1, ZCT2, and ZCT3 [38]). The most extensively studied of these are of the ORCA domain. The overexpression of ORCA3 in C. roseus hairy roots resulted in enhanced expression of several biosynthetic genes in the TIA pathway, including: Anthranilate Synthase (AS), Tryptophan Decarboxylase (TDC), 1-Deoxy-D-xylulose 5-phosphate synthase (DXS), Cytochrome P450 reductase (CPR), Geraniol 10-hydroxylase (G10H), Secologanin synthase (SLS), Strictosidine synthase (STR), Strictosidine b-D-glucosidase (SGD), and Desacetoxyvindoline 4-hydroxylase (D4H) [39]. Additionally, overexpression of ORCA3 in C. roseus cell suspensions enhanced the expression of STR, CPR, D4H, AS, DXS and TDC genes [32]. Overexpression of ORCA2 in C. roseus hairy roots showed enhanced levels of AS, STR, TDC, D4H, G10H, Tabersonine 16-hydroxylase (T16H), and Peroxidase 1 (PRX1) gene transcription [40].

STR catalyzes the first step in indole alkaloid biosynthesis and so much research has gone into studying how it functions as this is an indicator of upregulation of the TIA pathway [41]. Yeast one-hybrid screening an STR promoter indicated that STR is rapidly activated by ORCA2 and this expression is rapidly inducible with Jasmonic Acid (JA) and fungal elicitation, while ORCA1 is expressed constitutively and not involved in JA or fungal elicitation [31]. In addition to the ORCA transcription factors, CrBPF-1 can also bind to the STR promoter but at a different position. Some research suggests that CrBPF-1 may bind to the STR promoter after the ORCAs are bound [33]. CrMYC1 and CrMYC2 are transcription factors that have a basic helix-loop-helix structure and have shown evidence of activation via JA and fungal elicitation. The increase in mRNA levels of CrMYC1 in response to JA and fungal elicitation indicates that it validates the

expression of the STR gene [34]. Furthermore, CrMYC2 is thought to act upstream by activating the transcription of the ORCA transcription factors [35].

While ORCA2, ORCA3, CrBPF-1, CrMYC1, and CrMYC2 are shown to activate the transcription of genes in the TIA pathway, they have also shown to act as repressors of some TIA genes. ZCT1, ZCT2, and ZCT3, are activated by ORCA1 and ORCA2. The ZCTs have shown to repress the activation of the AP2/ERF domain of the ORCAs and activity of the promoters of TDC and STR [38]. The complex interactions between different transcription factors modulate the transcription of biosynthetic genes and activation level of other transcription factors in response to environmental signals. This effectively functions as a dynamic control system for TIA biosynthesis.

The last of the well-studied Jasmonate responsive transcription factors in C. roseus are CrWRKY1 and CrWRKY2 [36], [37]. Overexpression of CrWRKY1 in C. roseus hairy roots caused increases in TDC transcripts as well as transcripts of the ZCT repressors while downregulating the transcription of ORCA2, ORCA3, and CrMYC2 regulators [36]. Overexpression of CrWRKY2 in C. roseus hairy roots increased the mRNA transcript levels of TDC, N-methyltransferase (NMT), Deacetylvindoline 4-O-acetyltransferase (DAT), and Minovincinine 19-hydroxy-O-acetyltransferase (MAT), and upregulated the transcription of ORCA2, ORCA3, CrWRKY1, ZCT1, and ZCT3 [37]. Figure 3 shows a regulation map of the TIA biosynthesis pathway.

Jasmonic Acid induction of the TIA pathway

Jasmonic Acid and Methyl-Jasmonic Acid (Me-JA) are derivatives of the active lipid-derived hormone Jasmonoyl-isoleucine (JA-Ile) [42]. Jasmonic Acid and its derivatives have been shown to act as a means of defense response in many species of

plants [43]. As mentioned earlier, in C. roseus, JA and its derivatives have been shown to upregulate the activity of ORCA2, ORCA3, CrMYC1, CrMYC2, CrWRKY1, CrWRKY2, ZCT1, ZCT2, and ZCT3 transcription factors. Due to the complex regulation of the TIA pathway by these transcription factors, it is of particular interest to investigate the timing of JA induced gene sequencing. The relative transcript levels of ORCA3 and its regulated genes: D4H, STR, G10H, TDC, and CPR, in C. roseus seedlings were shown to fluctuate differently 96 hours after administration with Me-JA on their leaves [44]. Additionally, the primary pathway of conjugation and deactivation of JA-Ile has been identified as a CYP450 mono-oxygenase [42]. This study investigates the alkaloid levels of incubated and non-incubated C. roseus leaf extracts harvested at various times after administration of a Clotrimazole, a CYP450 inhibitor, and JA to the leaves.

Materials and Methods

Chemicals

Jasmonic Acid (Sigma Aldrich, 1.5 & 0.5mM) were dissolved in Dimethylsulfoxide (Uvasol, 0.2% by volume) in ethanol. Clotrimazole (Acros Organics, 200μM) was dissolved in pure ethanol. Acetone was obtained from Fischer Scientific.

Incubated Leaf Extracts

Homogenized Leaf Suspensions

All of the leaves of three mature C. roseus vt. Vinca Cora plants were sprayed on the top and bottom with a solution of Jasmonic Acid until liquid dripped from the leaves. The plant was kept in an Innova 4430 Incubator Shaker kept at 26.5ºC and watered every-other day with 200mL of water. Three leaves from each plant were harvested at

times of 0, 0.5, 3, 6, 9, 12, 24, 48, 72, and 96 hours after Jasmonic Acid administration and flash frozen in liquid nitrogen. Samples were kept in a -20ºC freezer for 24 hours. A variable volume of extraction buffer (100mM tris-HCl, pH 7.6, 13μM β-mercaptoethanol) was added to each test tube. The amount of buffer added to each leaf was dependent on the fresh weight of the leaf. Leaves were projected to have a protein content of 5.5mg/g fresh weight and water content of 82.5% of the fresh weight based on preliminary Bradford protein assay and freeze drying trials. Extraction buffer was added to each sample to reach a projected protein concentration of 2g/L. The leaf samples were then ground in 15mL conical test tubes using a custom machined aluminum pestle. After grinding, the samples were centrifuged for five minutes at 15,000 RPM and 4ºC and the supernatant was transferred to a new test tube. These suspensions were vortexed for one minute and incubated in a 25ºC water bath for 120 minutes.

Acetone Protein Precipitation for Alkaloid Isolation

2.5mL of cold acetone (-20 ºC) was added to the suspensions to precipitate out the proteins and the mixture was incubated for 24 hours to ensure complete protein precipitation. The solutions were then syringe filtered through a 0.2μm filter and the filtrate was dried over Nitrogen. The dried samples were then freeze dried for three days, suspended in 0.5mL of pure Methanol, and syringe filtered through a 0.2μm filter for HPLC-UV analysis.

Extraction Efficiency

Tabersonine-fed Methanol Alkaloid Extraction

Three leaves of a mature C. roseus vt. Vinca Cora plant were excised and frozen at -20ºC for 24 hours. The leaves were freeze dried for three days, after which they were crushed in a test tube using a custom machined aluminum pestle. 5mL of Methanol was added to the crushed leaves along with 41.25µL of 2mM Tabersonine in ethanol/gFW of leaf. The samples were vortexed and soaked for 3 hours before being filtered through a 0.2µm filter and dried over Nitrogen. The dried samples were suspended in 0.5mL of Methanol and prepared for HPLC-UV analysis.

Tabersonine-fed Incubated Alkaloid Extracts

Six leaves of a mature C. roseus vt. Vinca Cora plant were excised and frozen at -20ºC for 24 hours. After freezing, 41.25µL of 2mM Tabersonine in ethanol/gFW of leaf was added to the remaining six leaves. The leaves were then subjected to the same protocol outlined in "Homogenized Leaf Suspensions"; except half of them were not subjected to incubation and 2.5mL of cold acetone was added immediately after leaf homogenization. These samples were then processed via the "Acetone Protein Precipitation for Alkaloid Isolation" protocol and subjected to HPLC-UV analysis.

Non-Incubated Samples

Clotrimazole and Jasmonic Acid Coupled Administration

Before each experiment, the plant was watered with 200mL of water and three leaves from a mature C. roseus vt. Vinca Cora plant were excised and flash frozen in liquid Nitrogen. 200µM Clotrimazole in ethanol was sprayed onto the top and bottom of

every leaf of the plant. Five minutes after Clotrimazole administration, 1.5mM Jasmonic Acid in ethanol was sprayed on the top and bottom of every leaf of the plant. Three leaves were excised and flash frozen in liquid Nitrogen at times of 0, 0.5, one, two, three, and four hours after Jasmonic Acid administration.

Methanol Alkaloid Extraction

The frozen leaves were freeze dried for three days, after which they were crushed in a test tube using a custom fit Aluminum pestle. 5mL of Methanol was added to the crushed leaves. The methanol/leaf mixture was vortexed and soaked for 3 hours before being filtered through a 0.2μm filter and dried over Nitrogen. The dried samples were suspended in 0.5mL of Methanol and prepared for HPLC-UV analysis.

Testing Conditions

Trials with experimental controls were done on the same plant one week after the previous experiment. The order under which the experimental groups were tested was: overall control (0mM Clotrimazole in ethanol and 0mM Jasmonic Acid), Clotrimazole control (200mM Clotrimazole and 0mM Jasmonic Acid), Jasmonic Acid control (0mM Clotrimazole and 1.5mM Jasmonic Acid), and the experimental group (200mM Clotrimazole and 1.5mM Jasmonic Acid).

HPLC-UV Analysis

The Waters HPLC system used consisted of a 1525 Binary Pump, a 717 plus Autosampler, and a 2996 Photodiode Detector. 20μL of the alkaloid suspensions were injected into a Phenomenex Luna C18(2) HPLC column (250mm x 4.6mm). For the first five minutes, the mobile phase was a 30:70 mixture of Acetonitrile:100mM Ammonium Acetate (pH 7.3) flowing at a rate of 1 mL/min. Over the next 10 minutes, the mobile

phase was linearly ramped to a 64:36 mixture, which was maintained for 15 minutes. For the next five minutes, the flow rate was linearly increased to 1.4 mL/min. During the following five minutes, the mobile phase ratio was increased to 80:20, and maintained for 15 minutes. The flow then returned to starting conditions and the column equilibrated. Standards of strictosidine, tetrohydroalstonine, serpentine, vindoline, catharanthine, ajmalicine, hörhammericine, lochnericine, tabersonine, vincristine, and vinblastine were used to determine alkaloid concentrations of the samples.

Theory

Mechanism of Jasmonic Acid

The timing of gene activation for Jasmonic Acid induced genes varies over the course of 96 hours. Figure 4 shows the expression patterns of six genes in the shoot tips of seedlings exposed to 0, 0.2, 1.0, and 2.0mM Methyl Jasmonic Acid. However, the anticancer dimers have been found to accumulate less in seedlings than in full grown plants [45]. Bernonville et al. proposes a mechanism of action of Jasmonic Acid [2]. This mechanism proposes that Strictosidine acts as a short term defense mechanism against folivorous attack, while Vincristine and Vinblastine act as a long term defense mechanism in the leaves surrounding the leaf undergoing folivorous attack. Figure 11 demonstrates this mechanism.

Incubation of Jasmonic Acid administered leaves

The spatial separation of the monomers vindoline and catharanthine within the leaf suggests that the production of the dimers is largely dependent on the mechanical breaking of the leaf [2], [7]. Incubating homogenized leaf samples in a protein extraction

buffer allows for exposure of the monoterpeniod pathway enzymes and the monomeric compounds vindoline and catharanthine to each other.

Inhibition of the Regulatory Pathway

The conjugation and deactivation of Jasmonic Acid Isoleucine (JA-Ile) has been shown to be primarily dependent on a Cytochrome P450 Monooxygenase, CYP94B3 (Figure 6). Some of the enzymes in the monoterpeniod pathway have been identified as Cytochrome P-450 enzymes as well, some of which are also upregulated by Jasmonic Acid [9]–[11]. Administration of a Cytochrome P-450 inhibitor to C. roseus leaves before administration of Jasmonic Acid provides prolonged exposure of Jasmonic Acid to the genes of C. roseus, increasing the amount of time the proposed long term defense mechanism lasts.

Results and Discussion

Incubated Homogenized Leaf Samples

Leaves of a mature C. roseus plant were sprayed with a solution of Jasmonic Acid and excised at varying times after JA administration. The leaves were ground in a test tube with a tris-HCl protein extraction buffer. Alkaloid levels of Serpentine, Vindoline, and Catharanthine are shown in Figure 5. The monoterpeniods strictosidine, tetrohydroalstonine, ajmalicine, hörhammericine, lochnericine, and tabersonine as well as the dimeric indole alkaloid compounds vincristine and vinblastine were not detected by the HPLC in any samples. All experimental groups show high variability and overall low alkaloid levels. In general, vindoline levels of JA administered leaves were lower with respect to the control and serpentine levels fluctuated among all experimental groups.

Increased levels of Catharanthine were seen in all experimental groups immediately after JA and control solution administration, and these levels decreased sharply over the following 3 hours. Catharanthine levels gradually increased to the level of that immediately after JA administration by 12 hours after administration for the 0.5mM JA administered group, and 96 hours for the 1.5mM administered group.

The leaf samples were homogenized to increase the interaction between the spatially separated monomers vindoline and catharanthine. Excising JA induced leaves at varying times allows for the plant to yield different transcription profiles due to the variance in gene induction timing from JA. This should theoretically lead to different levels of TIA metabolites, which are not seen in in Figure 5. While JA is known to be an activator of several biosynthetic genes in the TIA pathway, the genes are also susceptible to downregulation by G-box binding factors,GBF-1 and GBF-2 [33], and ZCT1, 2, and 3[38]. Therefore, the expression patterns of TIA genes are the result of a potentially highly complex relationship between multiple activators and repressors which is outlined in Figure 3.

However, previous studies have found systematic changes in the alkaloid content of hairy root cultures and seedlings treated with JA or MeJA spray respectively[39], [46]. In contrast, no systematic changes of vindoline and catharanthine content of flowering C. roseus plants were found from 0 to 96 hours after treatment with MeJA [47]. Figure 3 shows the complex regulation scheme in C. roseus, the transcription factors directly affected by MeJA are CrWRKY2 and the ZCT1, 2, and 3. Figure 4 shows that ORCA3 is also directly or indirectly affected by MeJA [44]. Altogether, the induction patterns of TIA genes by JA and MeJA cannot be assumed to be analogous, and the studies of MeJA

administration cannot necessarily be directly compared to studies of JA administration. Figure 6 shows the major pathways for biosynthesis and conjugation and deactivation of JA-Ile; JA-Ile is the active form of the JA hormone [42]. Uncharacterized steps of JA biosynthesis from MeJA may exist which would suggest that MeJA and JA use the same mechanism of induction of the TIA pathway. MeJA may also regulate the transcription factors directly, such as is suggested for other JA conjugates in Figure 6.

Another difference between the present study and the studies showing systematic changes in alkaloid content in response to JA or MeJA treatment is the age of the organism. Seedlings and hairy root cultures are very young celled organisms whereas in the present study and the study done by Qifang Pan et al (2010) uses mature C. roseus plants. The proposed mechanism of JA by Bernonville et al suggests that folivorous attack elicits a long term defense response in neighboring leaves via signals through the stem of the leaf undergoing attack. Seedlings have less adjacent leaves to receive signals from, potentially causing a more uniform response to JA administration. Hairy roots start as infected, germinated seeds. The treatment of hairy roots to JA involves growing the roots in liquid cultures containing it. Having JA in the growth medium provides constant exposure to JA which can conflict the timing of genes, or cause a runaway response due to continuous exposure, potentially producing a more uniform response in alkaloid levels. The reduced level of differentiation into many types of cells for seedlings and hairy roots prior to exposure to JA can potentially cause a more uniform response compared to mature plants.

The mechanism of JA proposed by Bernonville et al also suggests that still unknown signals may control the increased biosynthesis of MIAs in distal, newly formed

leaves. The mechanism suggests that JA signals from existing leaves travel through the stem and create systematic changes in shoot gene transcription, resulting in transcriptional changes in the developing leaves, demonstrated in Figure 11. A particular consideration with this proposed mechanism is the higher concentration of auxin in shoot apical meristem tissue [48]. Auxin is known to be an inhibitor of select genes in the TIA pathway; this in combination with JA signals from mature leaves to the shoot tips suggests that auxin localization could play a larger role in TIA regulation than originally thought. Furthermore, if JA affects the transcripts of newly formed leaves, the levels of catharanthine and vindoline in newly formed distal leaves may have more significant responses to JA administration than the fully developed leaves tested in Figure 5.

Extraction Efficiency

Figure 7 and Figure 8 show chromatograms, at 254 and 329nm respectively, of alkaloids extracted via: Methanol extraction, non-incubation extraction, and incubated extracts. Alkaloids extracted via methanol extraction were allowed to soak in methanol for 3 hours. The methanol was dried over nitrogen and the alkaloids were suspended in a smaller volume of methanol and prepared for HPLC analysis. Alkaloids extracted via non-incubation extraction were ground in a test tube with a machine fit aluminum pestle in the presence of a tris-HCl protein extraction buffer and the proteins were immediately precipitated out with cold Acetone. The alkaloids were dried, and suspended in methanol for HPLC analysis. Alkaloids extracted via incubation were allowed to incubate in a water bath at 25ºC for 2 hours before protein precipitation and HPLC preparation. Only one of the triplicate trials is shown for each method. Tabersonine was found to elude the column at the same time as another, unknown, metabolite.

Due to the inability of Tabersonine to be isolated by the column, the extraction efficiency for each method of alkaloid extraction could not be determined. However, it is evident in Figure 10 that the Methanol extraction method yielded far higher amounts of serpentine, vindoline, and catharanthine compared alkaloids extracted in the presence of the tris-HCl protein extraction buffer.

The UV-spectrum of the molecule that eludes the column at the same time as Tabersonine is very similar to that of Tabersonine. It should be noted that Tabersonine eluded the column 31 minutes after injection. Typically, under these conditions Tabersonine eludes the column 38 minutes after injection. Despite being unable to calculate extraction efficiency of each method, the dependence on incubation of the samples can still be qualitatively seen in Figure 8. The unknown in Figure 8b dramatically decreases from 0.003AU to 0.001AU after 2 hours of incubation (Figure 8c). Furthermore, the peak at 12 minutes increases from 0.003AU to 0.005AU after incubation. These results combined with the complete absence of dimer formation indicate that incubation is an ineffective method of inducing dimer formation. One explanation is that digestive secretions from caterpillars participate in TIA biosynthesis of the toxic dimers, or provide cofactors for enzymatic steps. Uncharacterized steps and the complex regulation of the pathway do not discount this hypothesis.

Jasmonic Acid and Clotrimazole Administered Methanol Extracts

Alkaloid levels of Serpentine, Vindoline, and Catharanthine are shown in Figure 9. The monoterpeniods strictosidine, tetrohydroalstonine, ajmalicine, hörhammericine, lochnericine, and tabersonine as well as the dimeric indole alkaloid compounds vincristine and vinblastine were not detected by the HPLC in any samples. All

experimental groups show high variability and overall higher levels of alkaloids than in homogenized leaf suspensions, as shown in Figure 10. A similar result in Catharanthine levels is seen in the 1.5mM experimental group from the homogenized leaf suspension trial (Figure 5c) to the Methanol extraction trial (Figure 9c) – an initial spike in catharanthine levels immediately after JA administration followed by an immediate decrease. Clotrimazole experimental control groups showed significant increases in Catharanthine levels up to 2 hours after administration, however, JA administered groups also administered Clotrimazole showed no such trend. Serpentine levels amongst all experimental groups stayed relatively constant, except for the clotrimazole and JA administered group 4 hours after JA administration. Vindoline levels were lower at all times for leaves administered Clotrimazole and JA; and the standard deviation is lower than leaves only administered JA, and dramatically lower than the overall control group. Notably, the overall control group shows the most error at most time points for the majority of measured compounds. In general, the data of Clotrimazole and Jasmonic Acid administered samples showed the smallest amount of variability at each time point for all measured compounds. Six days after JA administration to plants also administered clotrimazole, the plant showed significant signs of wilting and distress in comparison to other C. roseus plants subjected to the same environmental conditions. However, a leaf sample was not taken.

Clotrimazole was administered to the leaves prior to JA in an attempt to inhibit CYP94B3, the primary enzyme responsible for the deactivation of JA-Ile, in an attempt to prolong the effects of JA and further conflict the timing of gene sequencing. Due to the fact that multiple genes in the TIA pathway are also cytochrome P450 enzymes, the

pathway was inhibited. The first enzyme in the pathway going towards vindoline from tabersonine is a cytochrome P450 enzyme, inhibition of this enzyme could cause the initial decrease from original vindoline levels in the group administered clotrimazole and the JA control. However, tabersonine levels did not increase in response to inhibition of T16H, indicating that flux through an alternative pathway could have occurred. The resulting increase in vindoline levels could be due to dissociation of the inhibitor from this enzyme, restoring vindoline levels to that before JA control administration. The experimental groups administered JA showed increased control over vindoline levels, and the group administered clotrimizole and JA did not show an initial decrease in vindoline levels. This confirms that JA regulates the TIA pathway, but the lack of decrease in vindoline levels indicates that newly translated T16H transcripts maintained the original vindoline levels. However, this does not agree with the mechanism outlined in Figure 11 as vindoline is proposed to be a long term defense response. In this mechanism, JA is thought to travel through the stem to adjacent leaves, not on the surface of the leaf. Administration of JA directly to the leaf surface could cause erroneous levels of vindoline to appear due to improper signaling. However, transcription of JA induced genes indicates that the inhibition of CYP94B3 was not achieved as originally intended.

The overall decrease in variance of vindoline levels in JA administered groups confirms that JA is a regulator of the TIA pathway, and the absence of significant changes in comparison to the overall control and original levels supports the mechanism outlined in Figure 11. Previous studies [47] reporting no changes in vindoline levels 96 hours after exposure to JA indicate that vindoline levels may be more dependent on other regulators.

Serpentine levels were largely unaffected in all experimental groups in Figure 9. Additionally, the total serpentine content was significantly less than that of vindoline and catharanthine in all experimental groups. This suggests that the branch of the TIA pathway leading to serpentine is not responsive to JA; although the absence of the serpentine precursor, ajmalicine, brings this hypothesis into question. The data in Figure 9 also supports the hypothesis that the uncharacterized steps toward serpentine are most likely not cytochrome P450 enzymes. The serpentine levels in Figure 9 are much more stable than the levels in Figure 5. Compared to catharanthine and vindoline, serpentine appears to be much more sensitive to incubation.

Leaves administered only clotrimazole showed significant increases in catharanthine content from 30min to 2 hours after administration. Considering that catharanthine is known to accumulate on the leaf surface, the location to which clotrimazole was administered, there are multiple potential reasons that could cause its delayed accumulation. One explanation is that catharanthine is a feedback inhibitor to a cytochrome P450 enzyme. Another possibility is that since the precursor to catharanthine is also a precursor to tabersonine, for which there are already 2 known cytochrome P450 enzymes, inhibition of the alternative branch of the pathway caused catharanthine accumulation, however, increases in tabersonine levels challenge this proposal. Cells responsible for the production of catharanthine are contained within the leaf; diffusion of clotrimazole through the different cells within the leaf could cause inhibition of a potential cytochrome P450 enzyme responsible for facilitating the secretion of catharanthine to the leaf surface, causing catharanthine to accumulate within the leaf and on the surface, increasing overall catharanthine levels. All experimental groups

administered clotrimazole also showed significantly less catharanthine accumulation immediately after administration, even when compared to the overall control. The absence of an initial spike in catharanthine content also indicates that clotrimazole inhibition is taking place and could be a similar result to that of vindoline inhibition previously discussed.

The same initial spike in catharanthine levels is seen in Figure 9b as is seen in Figure 5b, for groups administered 1.5mM JA; however the gradual decrease is not nearly as dramatic in methanol extracted samples. The initial spike in catharanthine in both groups is not characteristic of the mechanism proposed by Bernonville et al [2]. However, direct exposure to JA on the leaf surface may cause a different response than JA signals from neighboring leaves, as is proposed in Figure 11, especially in the case of catharanthine because it is located on the leaf surface. The resulting stabilization of catharanthine levels in the JA administered group is to be expected, as the initial spike may not have been intended at all with this proposed explanation. Leaves administered JA after clotrimazole did not show a spike in catharanthine levels, but followed the same trend as vindoline – little changes in alkaloid content with low levels of variation. If the proposed explanation for vindoline is true, it may also be the case for catharanthine as well. Direct administration of JA to the leaf surface as opposed to through the stem could cause an unintended spike in catharanthine and vindoline levels. This response appears to be dependent on cytochrome P450 enzymes which were inhibited by clotrimazole. Taken together, this supports the mechanism proposed by Bernonville et al. as vincristine and vinblastine being long term defense mechanisms against folivorous attack [2].

Conclusion

Jasmonic Acid administration to the leaves of mature C. roseus plants fails to produce the anticancer compounds vincristine and vinblastine from 0-96 hours after administration. Potentially conflicting signals from regulators in the pathway cause nonsystematic responses in alkaloid levels. Incubation of leaf extracts failed to have a significant effect on catharanthine and vindoline levels despite evidence of changes to other TIA levels. Clotrimazole decreased the variance of alkaloid levels when used in combination with JA, but caused alternative alkaloid accumulation when used with a control. Further work into identifying the regulation pathway of C. roseus alkaloid production is necessary to understand the increased regulation due to coupled clotrimazole and JA administration.

Future Works

Additional evidence is necessary to further confirm the mechanism of JA. A particular investigation into the dependence of JA exposure to seedlings and the resulting alkaloid levels in newly formed distal leaves compared to unadministered seedling distal leaves could further confirm the mechanism proposed by Bernonville et al [2]. Additionally, administering JA to one leaf instead of all of the leaves of a mature plant could produce more systemic responses in alkaloid levels of administered and distal leaves.

Comparisons with MeJA and JA administered hairy roots and seedlings are necessary to identify potential gene regulation differences. These results would confirm

the ability of JA conjugates other than JA-Ile to regulate transcription factors and gene transcription as proposed by Koo and Howe [42].

To more accurately see the effects of prolonged exposure of JA in C. roseus, gene editing technologies can be utilized to silence CYP94B3. This would confirm or deny the increased control of TIA levels in response to JA coupled with clotrimazole to prolonged JA exposure as opposed to alternative inhibition of metabolic or regulatory pathways.

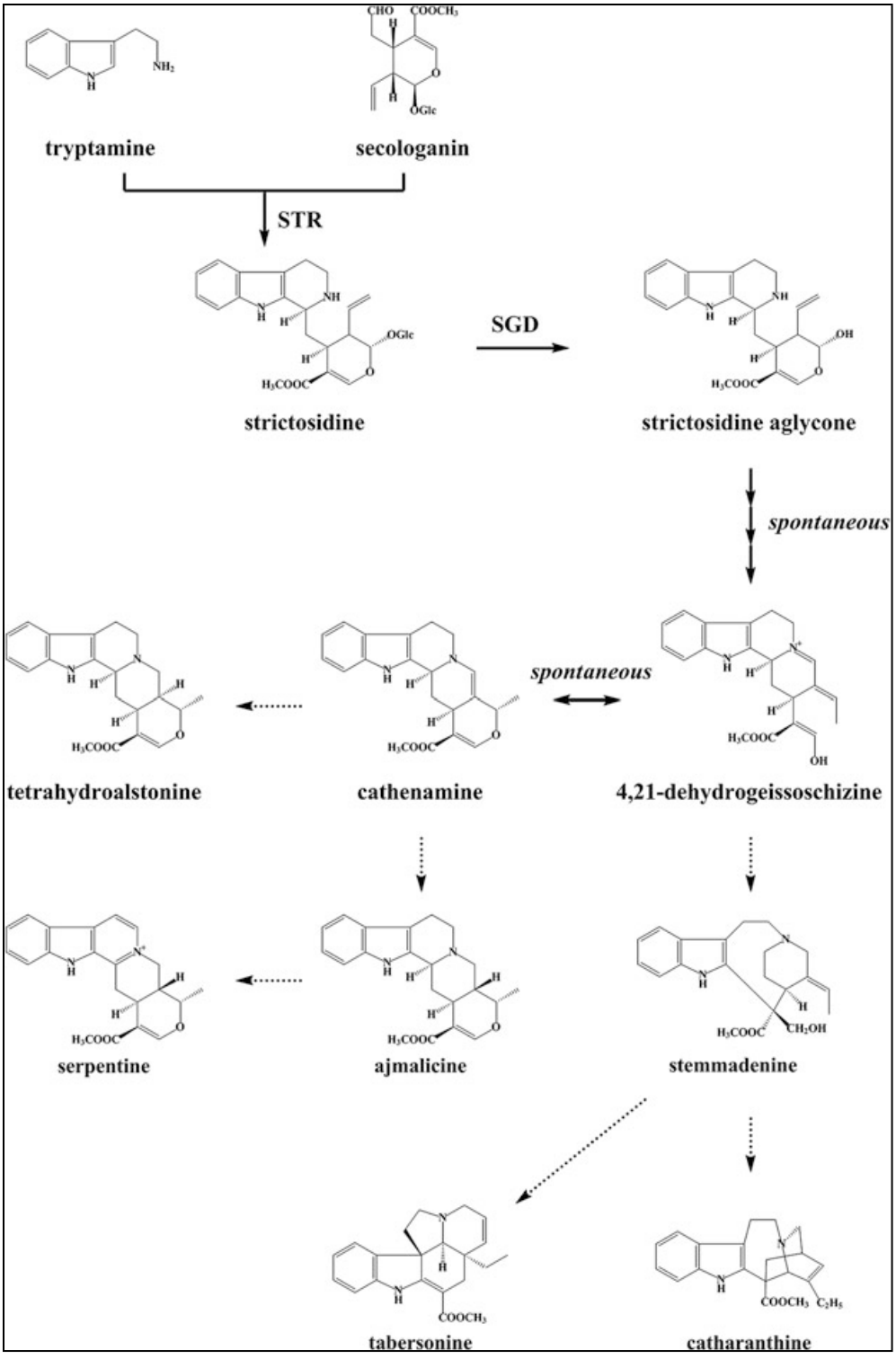

Figure 1 Taken from [5], The main branches of the downstream TIA biosynthesis pathway. STR: strictosidine synthase; SGD: strictosidine b-D-glucosidase. Dashed arrows indicate uncharacterized steps.

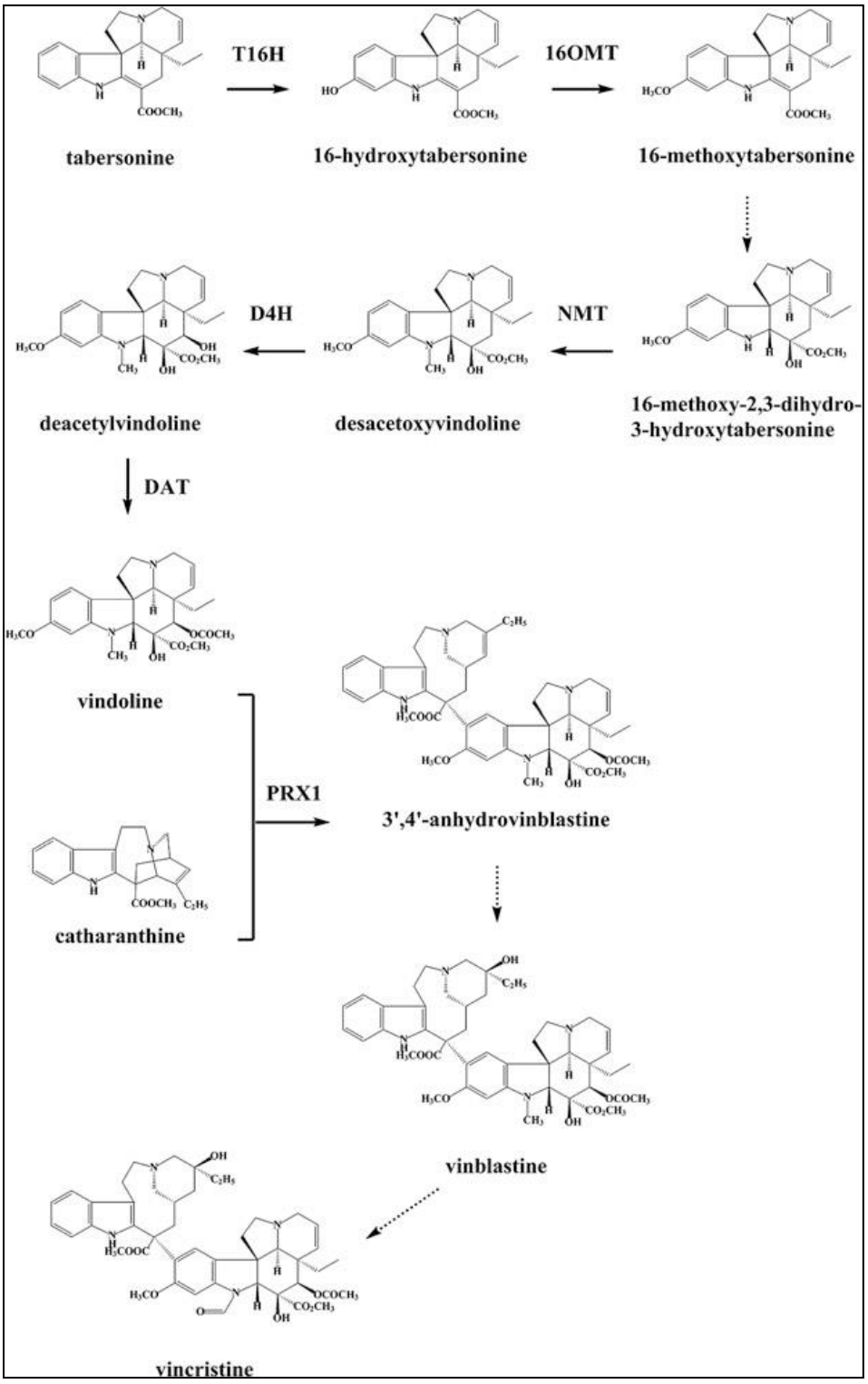

Figure 2 Taken from [5], The main branches of the downstream TIA biosynthesis pathway. STR: strictosidine synthase; SGD: strictosidine b-D-glucosidase. Dashed arrows indicate uncharacterized steps.

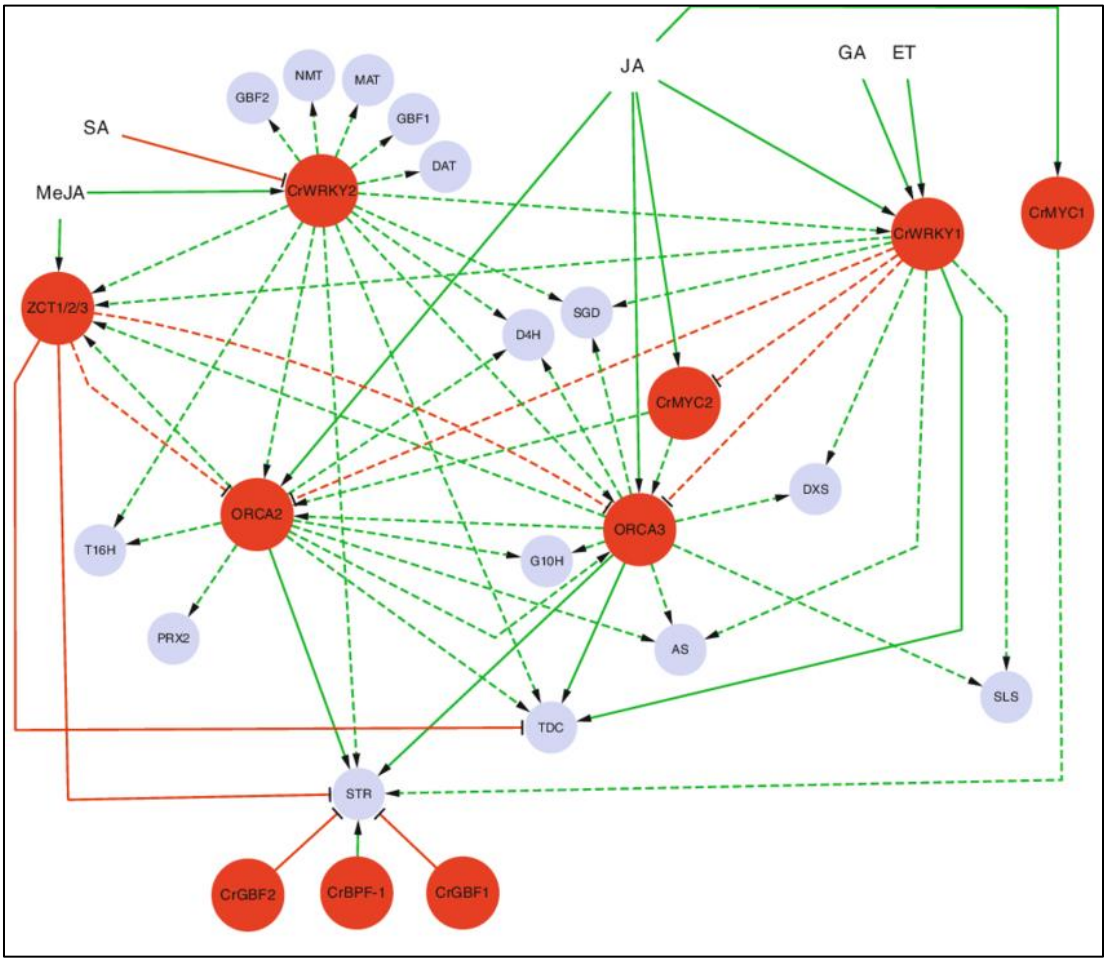

Figure 3 Taken from [5]. A regulation map of the TIA biosynthesis pathway. Nodes with empty backgrounds represent signaling molecules; nodes with grey backgrounds represent genes in the TIA pathway; nodes with red background represent transcription factors. Solid lines indicate direct interactions; dashed lines represent potentially direct or indirect interactions. Red lines indicate repressive action and green lines indicate activation. Note that the hormone auxin isn't present in the diagram.

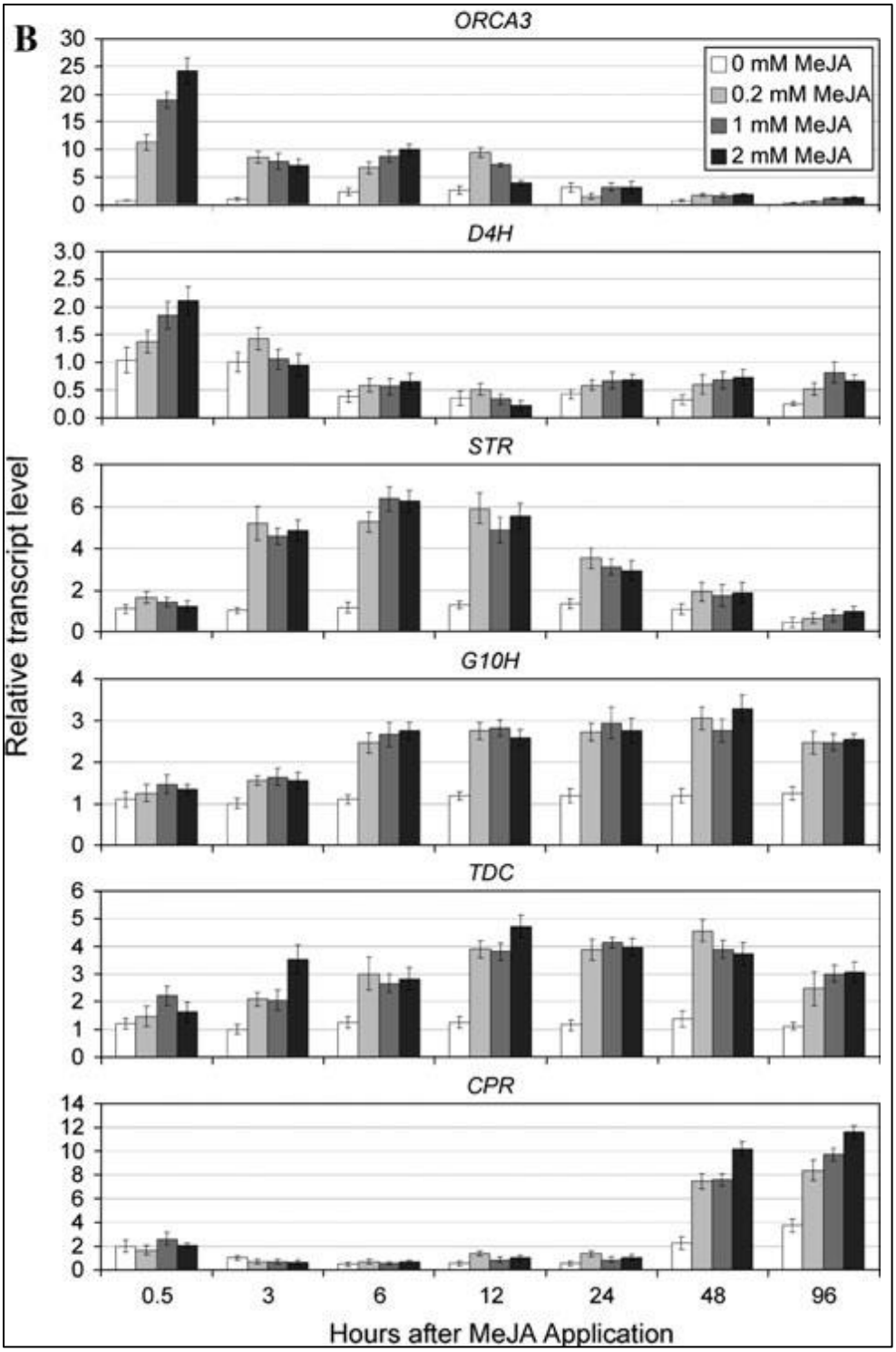

Figure 4 Taken from [44] . Relative transcript levels of ORCA3 and some of its regulated genes in shoot tips of seedlings after administration of varying concentrations of MeJA spray in 0.2% DMSO in ethanol.

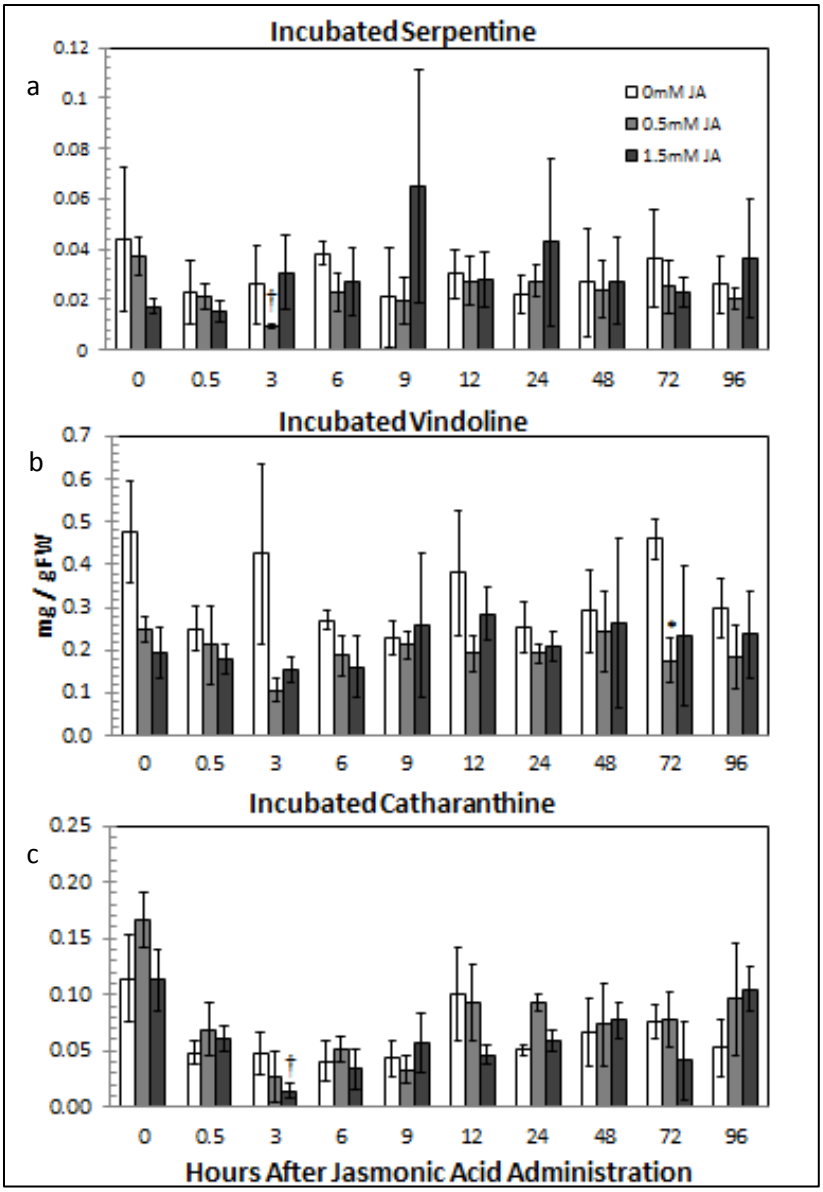

Figure 5 Serpentine, Catharanthine, and Vindoline levels of incubated, homogenized leaf suspensions following JA administration. Mature C. roseus plants were sprayed with solutions of 0, 0.5, or 1.5mM JA in 0.2% dimethyl sulfoxide (DMSO) in ethanol. Leaves were excised 0, 0.5, 3, 6, 9, 12, 24, 48, 72, and 96 hours after JA administration. The leaves were flash frozen in liquid nitrogen and homogenized with a variable amount of tris-HCl buffer to achieve a target protein concentration of 2.0g/L. Proteins were precipitated by adding 2.5mL of cold acetone. The mixtures were filtered through a 0.2µm syringe filter, dried over Nitrogen freeze dried for 3 days, and suspended in 0.5mL of methanol for HPLC-UV analysis. Bars indicate the standard deviation, n = 3. (* indicates a p value below 0.05 with respect to the overall control at the same time, and † indicates a p value below 0.05 with respect to the data at the previous time point in the same experimental group)

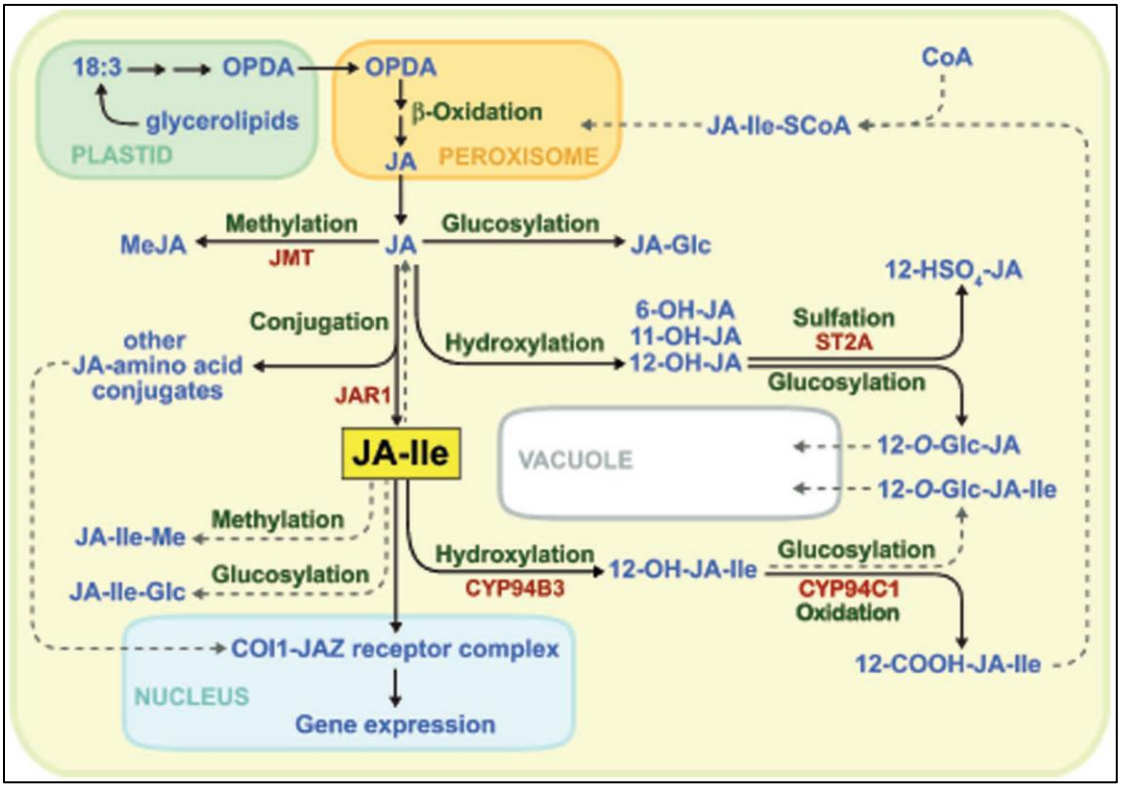

Figure 6 Taken from [42], major pathways for the biosynthesis and conjugation of JA-Ile. Solid lines indicate confirmed biochemical pathways, whereas dashed lines denote hypothetical pathways for which there is little or no evidence.

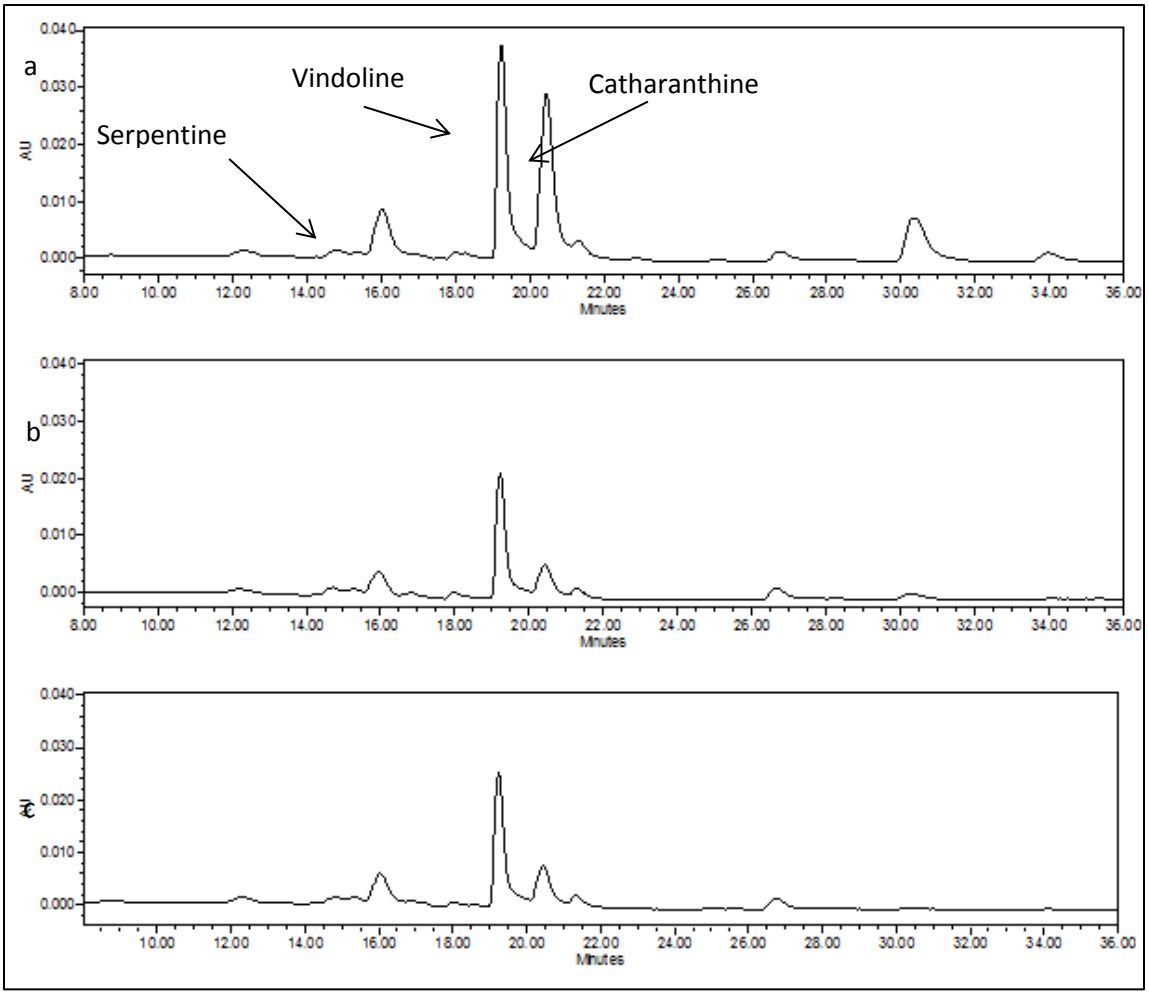

Figure 7 254nm chromatograms comparing extraction protocols of leaf samples fed Tabersonine. The top chromatogram contains alkaloids from Methanol extracted leaves, the second chromatogram contains alkaloid distributions from non-incubated leaf suspensions, and the bottom chromatogram contains alkaloid distributions from incubated leaf suspensions. Each sample was administered 4.3µL of 2mM Tabersonine prior to alkaloid extraction.

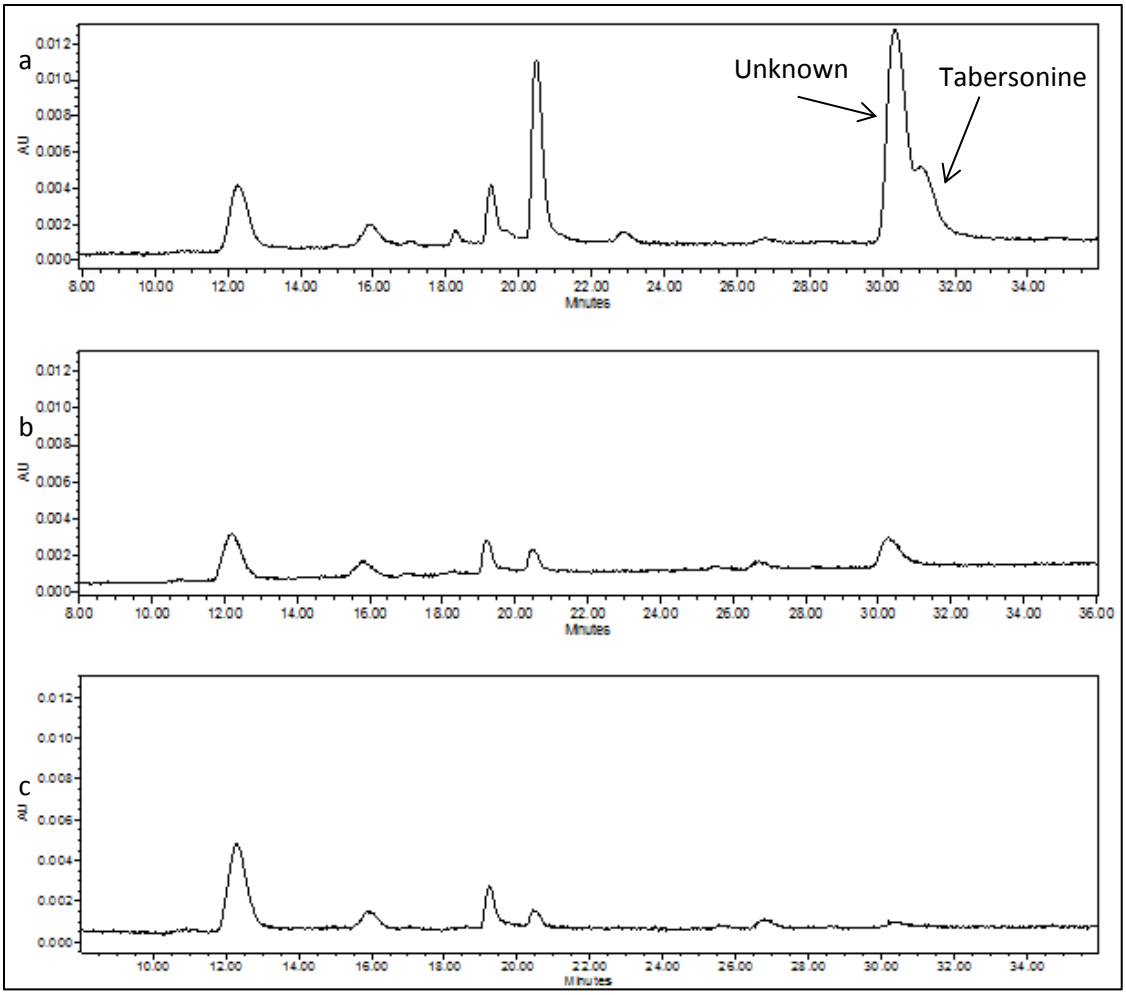

Figure 8 329nm chromatograms comparing extraction protocols of leaf samples fed Tabersonine. The top chromatogram contains alkaloids from Methanol extracted leaves, the second chromatogram contains alkaloid distributions from non-incubated leaf suspensions, and the bottom chromatogram contains alkaloid distributions from incubated leaf suspensions. Each sample was administered 4.3µL of 2mM Tabersonine prior to alkaloid extraction.

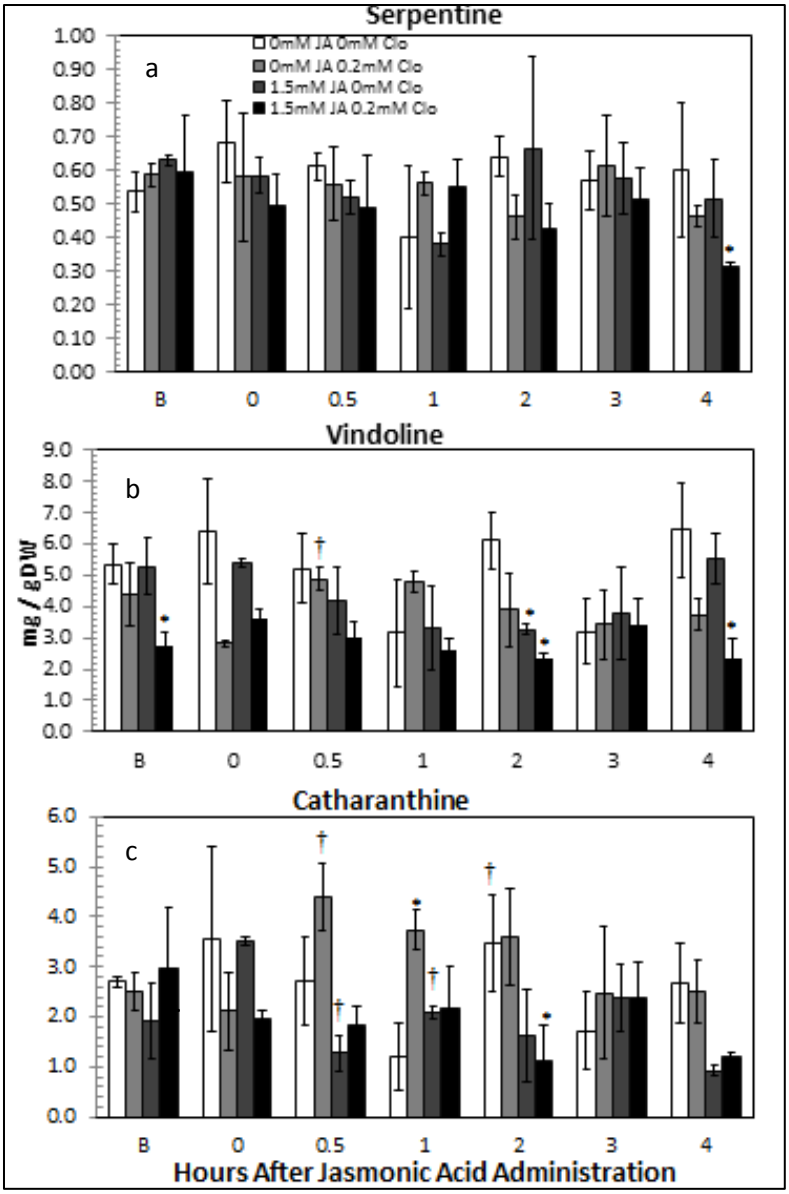

Figure 9 Levels of Serpentine, Catharanthine, and Vindoline after Clotrimazole and JA administration. Mature C. roseus plants were sprayed with solutions of 0 or 0.2mM Clotrimazole in ethanol and 0, or 1.5mM JA in 0.2% dimethyl sulfoxide (DMSO) in ethanol. Leaves were excised after Clotrimazole administration but before JA administration, and at 0, 0.5, 1, 2, 3, and 4 hours after JA administration. The leaves were flash frozen in liquid nitrogen and freeze dried for 3 days. The leaves were crushed in a test tube using a custom fit aluminum pestle and alkaloids then soaked in 2.5mL of Methanol for 3 days. The leaf/Methanol mixtures were filtered through a 0.2µm syringe filter, dried over Nitrogen, and suspended in 0.5mL of methanol for HPLC-UV analysis. Bars indicate the standard deviation, n = 3. (* indicates a p value below 0.05 with respect to the overall control at the same time, and † indicates a p value below 0.05 with respect to the data at the previous time point in the same experimental group)

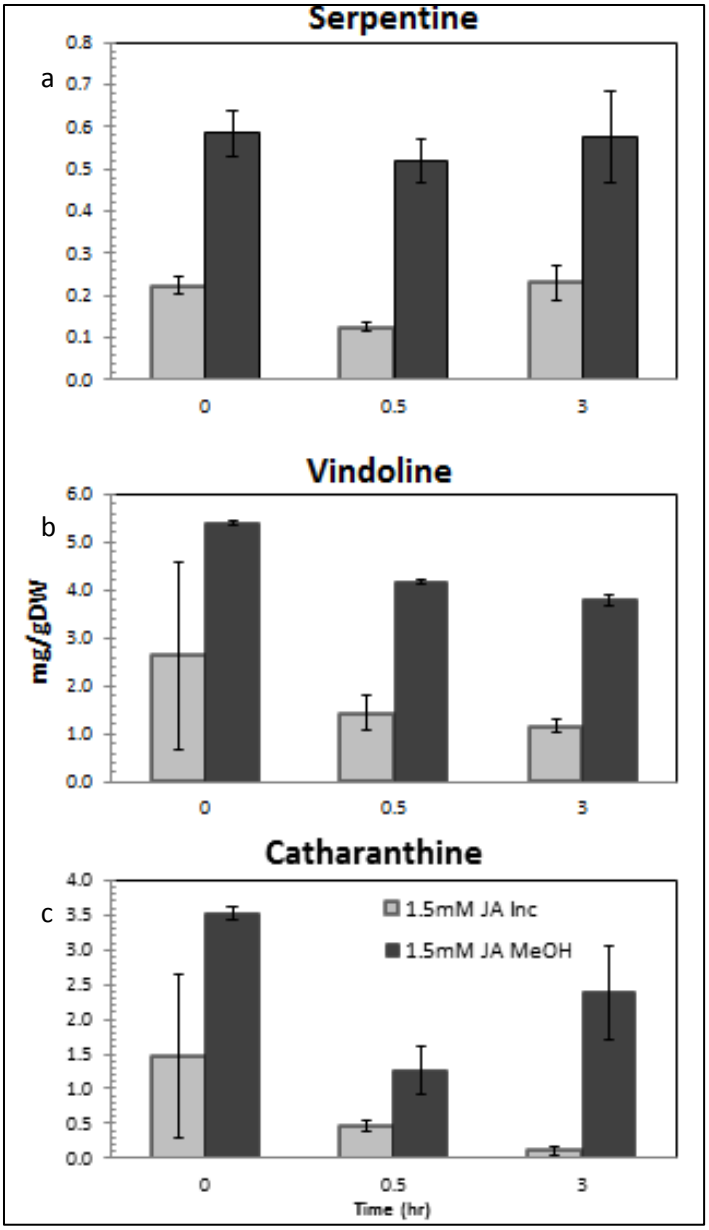

Figure 10 Levels of Serpentine, Vindoline, and Catharanthine in mature C. roseus leaves after administration of 1.5mM JA in 0.2% dimethyl sulfoxide (DMSO) in ethanol to the entire plant extracted using incubation and methanol extraction methods; reformatted data from Figure 5 and Figure 9. Dry weights of incubated samples approximated from 80% liquid content in fresh leaves based on preliminary trials. Bars indicate the standard deviation, n = 3.

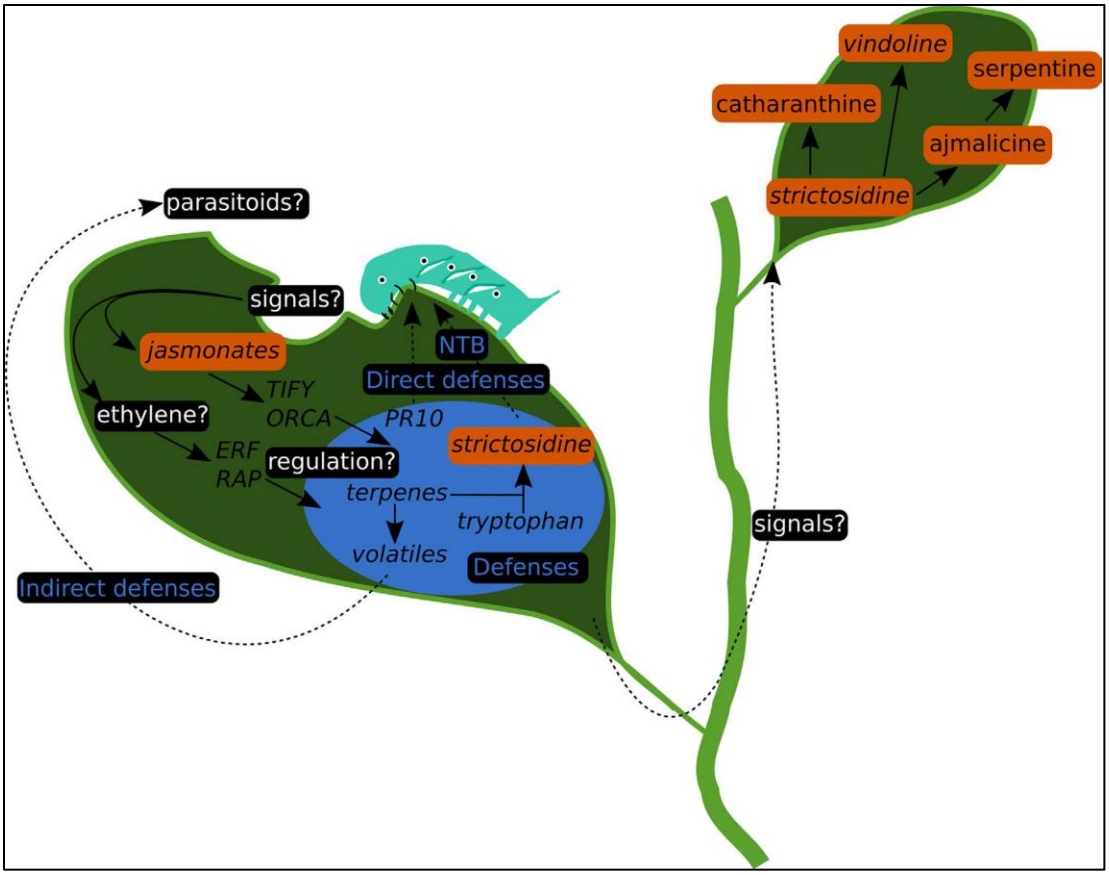

Figure 11 Taken from [2], molecular events associated with folivory in C. roseus; evidence supported by studies of Manduca sexta larvae being fed a diet of C. roseus. M. sexta was fed a diet of C. roseus for 72 hours. During the first 2 hours M. sexta was able to consume substantial amounts of C. roseus but feeding progressively decreased until total arrest after 72 hours. Orange boxes supported by compound measurements, italics supported by gene expression measurements. Nuclear Time Bomb (NTB). NBT involves a massive production of strictosidine aglycone, an efficient protein-cross-linker acting as a direct defense against folivory. TIFY are a class of Jasmonate repressive proteins; ORCA, ERF and RAP are plant pathogen defense transcriptional regulators; PR10 is a class of pathogenesis related proteins.

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