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Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus

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Chapter 1

General introduction

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Introduction

Plants produce an extensive array of secondary metabolites including terpenoids, phenolic compounds and alkaloids. These compounds play crucial roles in interactions between plants and their environment, and a significant number of them possess pharmacological activity in humans. *Catharanthus roseus* belongs to the Apocynaceae family and is extensively studied due to its ornamental value and therapeutic potential (Acharjee et al., 2022). The plant has been widely utilized due to its abundance of over 200 alkaloids most of which are monoterpenoid indole alkaloids (MIAs) (Das et al., 2020; De Luca et al 2014). Vinblastine and vincristine are common exemplars of MIA, resulting from the polymerization of catharanthine and vindoline (Fig. 1A) and can be applied for treating neuroblastoma, Hodgkin's disease, breast cancer, lung cancer, and chronic leukemia (Das et al., 2020; Dhyani et al., 2022; Van der Heijden et al., 2004). Vinblastine and vincristine are only biosynthesized in *C. roseus*, where their content is very low. Hence, enhancing the yield of MIAs is an interesting issue that has stimulated worldwide research efforts over the past few decades.

MIA biosynthesis in *C. roseus*

MIAs are a group of important secondary metabolites synthesized in *C. roseus*. Research on MIAs is largely primed by the medicinal activities of several of the compounds (Memelink and Gantet, 2007). The monomeric alkaloids ajmalicine and serpentine are utilized for their sedative effects in treating hypertension along with their antidiabetic activity (Datta et al., 2010; El-Sayed and Verpoorte, 2007). Two dimeric alkaloids vincristine and vinblastine are used in cancer treatment. In plants, MIAs are thought to play a role in defense responses. MIAs are only present in a limited number of plant families including Apocynaceae, Loganiaceae and Rubiaceae (Mohammed et al., 2021). The most significant advancements in molecular characterization of the pathway have been achieved using *C. roseus* (L.) G. Don (Madagascar periwinkle), that belongs to the Apocynaceae family (Kulagina et al., 2022).

The biosynthesis of MIAs in *C. roseus* relies on two precursors, secologanin derived from the MEP (Methylerythritol 4-phosphate)/seco-iridoid pathways and tryptamine from the shikimate/tryptophan pathways. The first MIA is synthesized by the condensation of tryptamine and secologanin (Fig. 1A) by the enzyme strictosidine synthase (STR) resulting in 3α(S)-strictosidine. Tryptamine, supplying the indole moiety of MIAs, is formed by decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (TDC). Secologanin, which provides the monoterpenoid part of the MIAs, is synthesized via the seco-iridoid pathway by several enzymatic conversions from geraniol. Hydroxylation of geraniol leads to the formation of 8-hydroxy-geraniol, catalyzed by geraniol 8-hydroxylase (G8O), which is the first step in the formation of secologanin (Collu et al., 2001). Geraniol synthase (GES), geraniol-8-oxidase (G8O), 8-hydroxygeraniol oxidoreductase (8HGO), iridoid synthase (IS), iridoid oxidase (IO), 7-deoxyloganetic acid glucosyl transferase (7DLGT) and 7-deoxyloganic acid hydroxylase (7DLH), are involved in conversion of geranyl-PP into loganic acid (Miettinen et al., 2014). Loganic acid O-methyltransferase (LAMT) is responsible for

conversion of loganic acid to loganin (Li et al., 2015). while secologanin synthase (SLS) catalyzes the last conversion of loganin into secologanin (Irmler et al., 2000). Strictosidine is subsequently deglucosylated by the enzyme strictosidine β-D-glucosidase (SGD) to form the highly reactive strictosidine aglycone. Additional enzymatic steps lead to the production of various MIAs through several distinct branches. For example, ajmalicine and serpentine, vindoline or catharanthine are produced by specific branches (Fig. 1A).
Dimeric alkaloids (vinblastine and vincristine) are formed by condensation of vindoline and catharanthine which is catalyzed by peroxidase (PRX1) (Sottomayor et al., 2004). Vindoline and dimeric alkaloids derived from vindoline are only present in chloroplast-containing plant tissues. Tabersonine is converted to vindoline by several steps which involves the action of seven enzymes spread over three cell types.
Similar to numerous specialized metabolites in various plant species, the biosynthesis of MIAs in *C. roseus* is controlled by the plant hormone jasmonate (JA) and the alkaloids are thought to function primarily in defense against insects and pathogens (De Geyter et al., 2012; Dugé de Bernonville et al., 2017; Goossens et al., 2016; Roepke et al., 2010; Wasternack and Hause, 2013).

Multicellular compartmentation of MIA in *C. roseus*

The MIA pathway in *C. roseus* is complexly organized both spatially and temporally, with specific steps distributed to various organs, tissues, and subcellular compartments. This structured coordination is particularly well-documented in the leaves (Kulagina et al., 2022). At the organ level, bisindole alkaloids such as vinblastine, vincristine, and vindoline are exclusively produced in the aerial parts of *C. roseus*, while catharanthine is found in all plant organs (Pan et al., 2016; Van der Heijden et al., 2004). At the cellular level, the initial seven steps of biosynthesis, from geranyl diphosphate (GPP) to the iridoid loganic acid, occur within the internal phloem associated parenchyma (IPAP) cells (Geu-Flores et al., 2012; Asada et al., 2013; Simkin et al., 2013; Miettinen et al., 2014; Salim et al., 2014). LAMT, SLS, TDC, and STR, the enzymes which catalyze the next four steps, occur in epidermal cells, leading to the production of strictosidine (Courdavault et al., 2014; St-Pierre et al., 1999). STR catalyzes the condensation of secologanin and tryptamine in the vacuole of leaf epidermal cells, leading to the formation of strictosidine. Strictosidine undergoes further deglycosylation by the enzyme SGD. Strictosidine aglycone is then converted to ajmalicine, tabersonine or catharanthine. The pathway leading to the precursors of anti-cancer compounds has been recently clarified. Two major enzymatic conversions of the strictosidine aglycone result in the formation of (a) ajmalicine and serpentine via cathenamine, and (b) vindoline and catharanthine via stemmadenine. These conversions occur through a series of steps across various cellular and subcellular compartments. (Tatsis et al., 2017; Caputi et al., 2018; Sharma et al., 2018; 2020; Qu et al., 2018; 2019; Colinas et al., 2021; Shen et al., 2024). The activity of PRX1 leads to dimerization of the monomeric MIAs vindoline and catharanthine to produce 3',4'-anhydrovinblastine, which directly serves as the precursor for vinblastine and vincristine (Costa et al., 2008; Van der Heijden et al., 2004) (Fig. 1B, 1C).

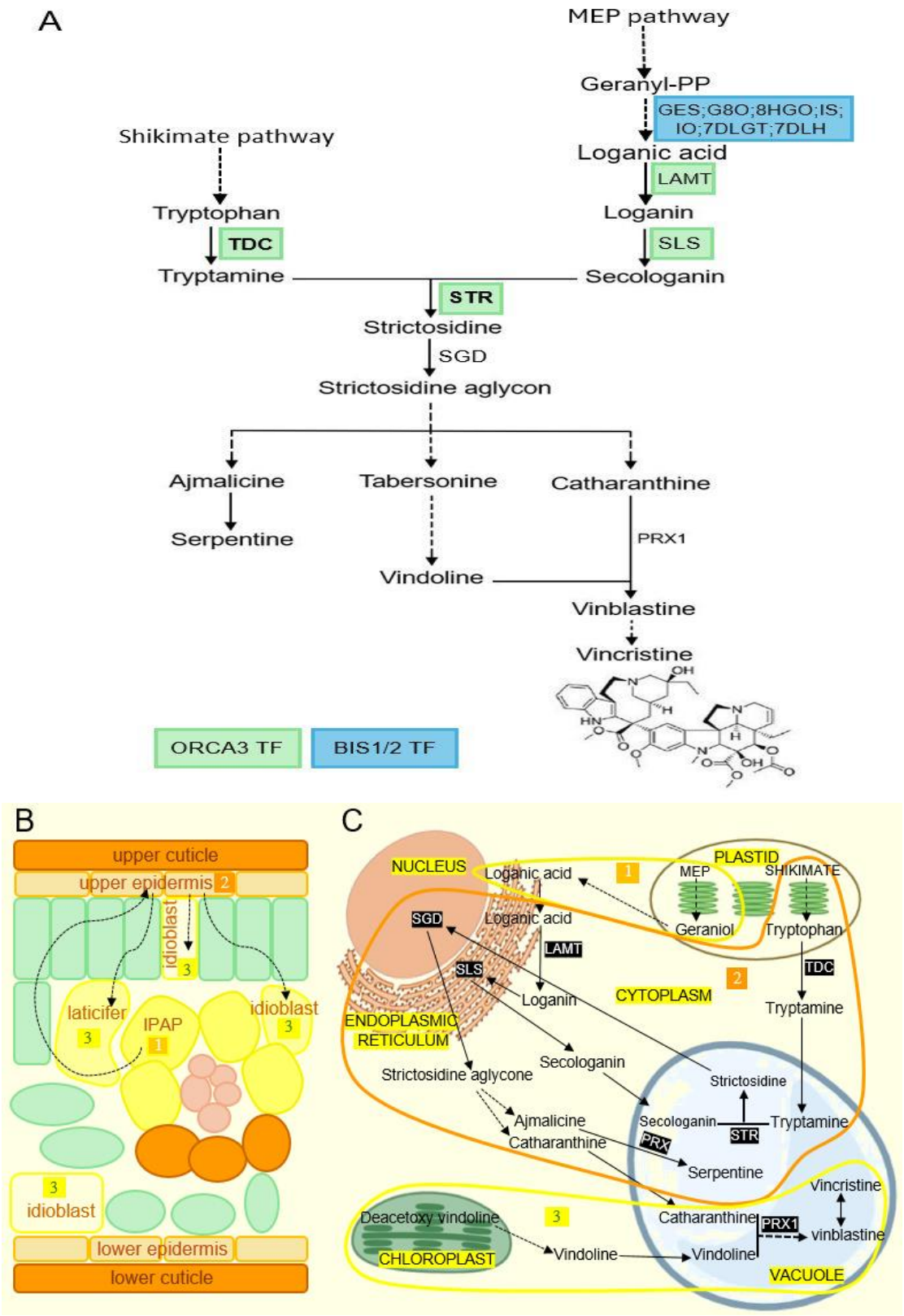

Figure 1. Schematic overview of the MIA pathway in *C. roseus* leading to the production of vinblastine and vincristine. (A) Pathway resulting in the production of shoot-specific MIAs in *C.roseus*. Genes regulated by BIS1 and ORCA are boxed in blue and green, respectively. (B) The MIA network and the established or hypothesized cellular and organ-specific expression patterns of the genes encoding MIA biosynthesis. MIA biosynthesis is localized into the three

distinct cell types shown in B and C. Numbers 1, 2, 3 correspond to internal phloem associated parenchyma (IPAP), epidermis and idioblast, respectively. (C) The MIA pathway, along with the established or proposed patterns of cellular and organ-specific expression of genes involved in MIA biosynthesis. Solid arrows represent single enzymatic steps and dashed arrows represent multiple enzymatic steps. ORCA3: octadecanoid-derivative responsive *Catharanthus* AP2-domain protein 3, GES: geraniol synthase, G8O: geraniol-8-oxidase, 8HGO: 8-hydroxygeraniol oxidoreductase, IS: iridoid synthase, IO: iridoid oxidase, 7DLGT: 7-deoxyloganetic acid glucosyl transferase, 7DLH: 7-deoxyloganic acid hydroxylase, LAMT: loganic acid O-methyltransferase, SLS: secologanin synthase, TDC: tryptophan decarboxylase, STR: strictosidine synthase, SGD: strictosidine β-D-glucosidase, PRX1: peroxidase (Modified from Colinas et al. (2021)).

Jasmonate signaling in MIA biosynthesis in *C. roseus*

Jasmonates (JAs), including jasmonic acid (JA) and some of its precursors and derivatives such as methyl-jasmonate (MeJA), are a group of well-known plant defense hormones. These signaling molecules control plant development and regulate responses to wounding, herbivore attack, necrotrophic pathogens and abiotic stresses (Zhou and Memelink, 2016; Li et al., 2021). Induction of secondary metabolite accumulation is a crucial defense response, which relies on JAs as a regulatory signal. Jasmonic acids are synthesized through the octadecanoid pathway (Huang et al., 2017). JA-L-isoleucine (JA-Ile) is the bioactive form of JAs (Sarafat Ali and Baek, 2020; Fonseca et al., 2009). All known biosynthesis genes involved in MIA production in *C. roseus* are induced by MeJA (Van der Fits and Memelink, 2000; Miettinen et al., 2014). Additionally, MeJA triggers gene expression in primary metabolism, resulting in the formation of MIA precursors, which shows the intense effect of JAs on plant metabolism by influencing gene expression.

Transcription factors involved in MIA biosynthesis in *C. roseus*

The biosynthetic pathway of MIAs is complicated and precisely controlled by transcription factors *in vivo*. Several key transcription factors have been identified that play crucial roles in controlling the expression of genes involved in the MIA biosynthetic pathway. These include members of the APETALA2/Ethylene Response Factor (AP2/ERF) family (Menke et al., 1999; Van der Fits and Memelink, 2000), basic helix-loop-helix (bHLH) proteins (Zhang et al., 2011; Van Moerkercke et al., 2015; 2016), WRKY (Van der Fits et al., 2000; Suttipanta et al., 2011), and C_2H_2 zinc finger proteins (Pauw et al., 2004; Rizvi et al., 2016). TFs typically control the transcription of multiple biosynthesis genes within a pathway. This ability makes them desirable tools for enhancing the production of secondary metabolites (Zhou and Memelink, 2016). Here, we discuss two classes of TF engaged in JA signaling cascade regulating the biosynthesis of several secondary metabolites which are relevant for this thesis.

bHLH transcription factors

Transcription factors that depend on the JAs signaling play a pivotal role in JAs signal transduction by controlling the expression of downstream genes through targeted binding to *cis*-acting elements within the promoters of these genes. The transcription factors from the bHLH family serve a crucial and frequently conserved function in the

plant kingdom (Goossens et al., 2017). The bHLH domain is composed of nearly 60 amino acids, where the N-terminal 15-20 basic amino acids are responsible for DNA binding. Additionally, there are two amphipathic alpha helices in the HLH region that facilitate the formation of homodimeric or heterodimeric complexes (Atchley and Fitch, 1997). The most extensively studied and multifunctional bHLH-domain transcription factor within the JAs signaling pathway is MYC2, responsible for regulating a specific set of genes responsive to JAs. In JA signaling in *Arabidopsis* a class of bHLH proteins, including MYC2, MYC3 and MYC4, serve as core TFs (Lian et al., 2017). The MYC family proteins possess a JID (JAZ-interacting domain) at their N-terminus, enabling them to interact with JASMONATE ZIM (JAZ) repressors and a transcription activation domain (TAD) (Zhang et al., 2015). In the absence of JA-Ile, JAZ proteins interact with the MYC proteins to inhibit their activity (Chini et al., 2007; 2009). Coronatine is a phytotoxin produced by *Pseudomonas syringae* that shares structural and functional similarities with JA-Ile. Coronatine-insensitive 1 (COI1) is an F-box protein that serves as the substrate-recruiting module of the Skp1/Cul1/F-box protein (SCF) ubiquitin E3 ligase complex which targets proteins for ubiquitination and subsequent degradation via the 26S proteasome (Sheard et al., 2010). In the presence of JA-Ile, a co-receptor complex forms, consisting of COI1, JA-Ile, and a JAZ protein. Degradation of JAZ proteins leads to the release of MYC TFs from repression (Goossens et al., 2015). Additional research has shown that MYC2 specifically bound to the G-box regions within the promoters of at least three *JAZ* genes, including *JAZ1*, *JAZ2* and *JAZ3* (Pauwels and Goossens, 2008; Niu et al., 2011; Chini et al., 2007), thereby stimulating their expression forming a negative feedback loop.

Analysis of *C. roseus* transcriptome databases (Gongora-Castillo et al., 2012; Van Moerkercke et al., 2013; Miettinen et al., 2014) to identify regulators of the iridoid biosynthesis pathway led to the discovery and characterization of the bHLH transcription factor Iridoid Synthesis 1/2 (BIS1/2). The BIS1 and BIS2 proteins exhibit 49% amino acid identity and 67% similarity overall. BIS1/2 are tightly co-expressed with MEP and iridoid pathway genes. They activate the expression of all genes encoding enzymes involved in the stepwise conversion of the universal terpenoid precursor, geranyl diphosphate, into the iridoid loganic acid (Van Moerkecke et al., 2015; 2016). Overexpression of BIS1/2 and a third related bHLH called BIS3 resulted in increased expression of the iridoid pathway genes, although to varying extents (Colinas et al., 2021).

ORCA AP2/ERF domain transcription factors in alkaloid metabolism

The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily is characterized by the AP2/ERF domain, which comprises about 60 amino acids and recognizes a GCC (AGCCGCC) motif for DNA binding (Mizoi et al., 2012). ORCA2 (octadecanoid-derivative responsive Catharanthus AP2-domain protein 2) was the first JA-responsive TF identified (Menke et al., 1999). Shortly thereafter, the related TF ORCA3 was discovered (Van der Fits and Memelink, 2000). The ORCAs occur in a

genomic cluster containing five *ORCA* genes (Paul et al., 2017; Singh et al., 2020), which all exhibit upregulation in response to MeJA (Yamada and Sato, 2021). MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is regulated by a transcription factor cascade including the bHLH TF CrMYC2 which operates upstream of ORCA2 and ORCA3, thereby activating their transcription and subsequently, the ORCAs control a subset of biosynthetic genes in the middle part of the MIA pathway including *TDC* and *STR* (Zhang et al., 2011).

It has been shown that redundancy and sub-functionalization of transcription factor families can coexist, affecting different target genes simultaneously. Regarding the ORCA cluster, it was observed that they redundantly activate pathway genes required for synthesizing common precursors, specifically the "early pathway genes". In contrast, the later steps of the MIA pathway are partially regulated by specific individual members. ORCA3, for example, is more specific to certain root MIA pathway genes, while ORCA6 appears to have a more limited effect on MIA pathway genes, except for a moderate up-regulation of the root MIA genes (Colinas et al., 2021). Overexpression of ORCA3 in suspension cells (Van der Fits and Memelink, 2000) or hairy roots (Li et al., 2013) or ORCA2 in hairy roots (Peebles et al., 2009) led to the upregulation of multiple genes encoding enzymes involved in primary and secondary metabolism and consequently in an increase in accumulation of MIAs. Overexpressing ORCA4 in hairy roots also resulted in a significant rise in MIA levels (Paul et al., 2020).

Examples of F-box proteins regulating TF activity

The controlled synthesis of new polypeptides and the accurate degradation of existing proteins regulate every aspect of a plant's life (Smalle and Vierstra, 2004). Maintaining stability or protein turnover is crucial for regulating the levels of essential proteins that play significant roles in plant growth and development (Gange et al., 2002; Wang et al., 2014). The ubiquitin-proteasome system (UPS) is the primary regulatory mechanism used by plants to manage cellular protein turnover, involving the coordinated action of three enzyme classes, E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 ligases (Williams et al., 2019; Saxena et al., 2023). These E3 enzymes can be grouped into various large families. SCF (Skp1-Cullin 1-F-box) ubiquitin-ligase complex is one of the major E3 type SCF, which is the most well-characterized and consists of four primary subunits: Skp1, Cullin, Rbx1, and F-box Protein (Deshaies, 1999; Potuschak et al., 2003; Xu et al., 2009) (Fig. 2). One of the major protein families among eukaryotes, F-box proteins (FBPs), are essential constituents of the multi-subunit SCF complex within E3 ligases. At the N-terminus region of F-box protein, there is a conserved domain of 40-50 amino acids which is responsible for interacting with the Skp1 subunit. Additionally, it includes one or more highly variable protein-protein interaction domains at the C-terminus, enabling specific substrate recognition (Guo et al., 2018).

F-box proteins participate in numerous biological functions by interacting with specific target proteins, which frequently results in the degradation of these targets via the proteasome pathway (Gagne et al., 2002).

The ubiquitin-proteasome system modulates diverse biological functions within the plant including circadian clock, photomorphogenesis, auxin, JA, gibberellins and ethylene hormone responses (Smalle and Vierstra, 2004; Chen et al., 2018; Nagels Durand et al., 2016; Wang et al., 2011; Qiao et al., 2009), quite often by modulating TF activity.

One way to negatively regulate TF activity is to degrade the protein via SCF-mediated ubiquitination and proteasome-mediated degradation. Conversely, it is also possible to degrade a repressor of the TF and thereby release the TF in an active form.

Here, the roles of the F-box proteins COI1 in JA signaling, EIN3-binding F-box protein 1 (EBF1) and EBF2 in ethylene signaling, and TIR1 in auxin signaling will be discussed.

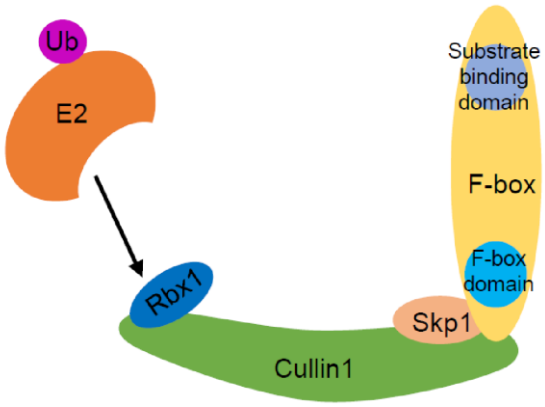

Figure 2. An SCF complex is composed of four primary subunits- Cullin1, Rbx1, Skp1, and an F-box protein. The Cullin1, Rbx1, and Skp1 subunits form the core ligase activity, with Rbx1 recruiting the E2 enzyme bearing an activated Ubiquitin molecule. F-box proteins play a vital role in delivering the right targets to the complex for ubiquitination.

COI1, a F-box protein in JA signaling

As previously mentioned, the bioactive form of the plant hormone JA, (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), and its structural and functional analog coronatine is recognized by COI1 and JAZ proteins, leading to the formation of a co-receptor complex (Williams et al., 2019) (Fig. 3). COI1 is classified as an F-box protein (FBP), which is usually a component of an SCF E3 ubiquitin ligase complex. The SCF^{COI1} complex is responsible for identifying and promoting the ubiquitination of the JAZ proteins, thus tagging them for degradation by the 26S proteasome (Sheard et al., 2010; Pauwels et al., 2015; Yan et al., 2018).

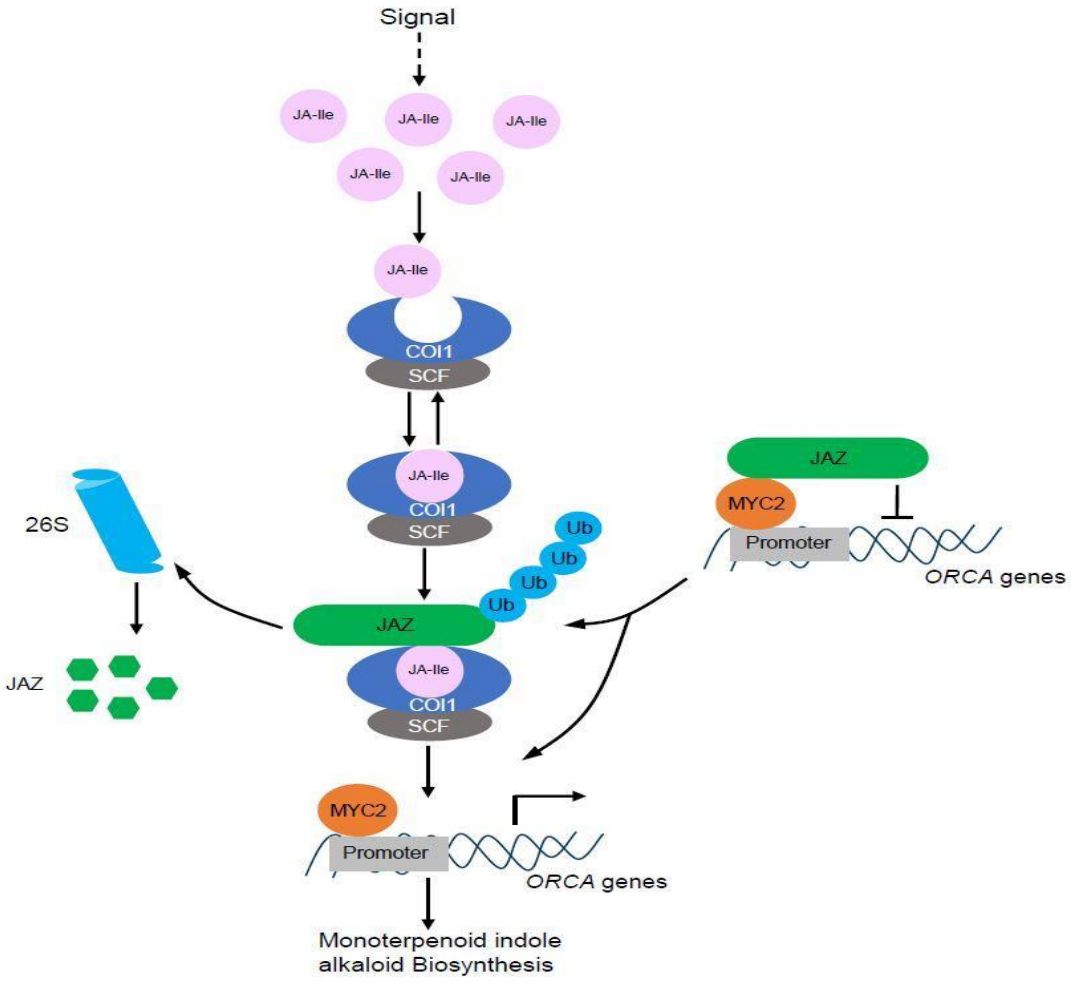

Figure 3. Model for JAs signal transduction leading to the expression of MIA biosynthesis genes in *C. roseus*. The F-box protein COI1 binds JA-Ile and successively recruits JAZ proteins for subsequent ubiquitination and degradation. JAZ repressors normally repress MYC2 activity among other TFs. Following JAZ degradation, MYC2 is liberated and induces transcription of *ORCA* genes. ORCAs then stimulate MIA production by increasing the transcription of genes encoding metabolic enzymes in the MIA pathway (modified from Zhai et al. (2017)).

EBF1/2, F-box proteins in ethylene signaling

The gaseous plant hormone ethylene regulates various aspects of plant developmental processes and stress responses (Zhu and Guo, 2008). The F-box proteins EIN3-binding F-box protein 1 (EBF1) and EBF2 play essential roles in the ubiquitination and degradation of the master transcription factors ethylene insensitive 3 (EIN3) and EIN3-like 1 (EIL1) within the ethylene response pathway (Hao et al., 2021). EBF1 and EBF2 play crucial roles in ethylene signaling, each contributing distinct but overlapping functions in the regulation of EIN3 stability (Binder et al., 2007). In the absence of ethylene, the Raf-like protein kinase constitutive triple response 1 (CTR1) phosphorylates the C-terminus of ETHYLENE INSENSITIVE2 (EIN2), which is a positive regulator of the pathway localized in the endoplasmic reticulum (ER) membrane, thereby preventing additional ethylene signal transduction. EIN2 is crucial for facilitating the accumulation of EIN3/EIL1 in response to ethylene and for the degradation of EBF1/2 (An et al., 2010). EBF1 and EBF2 in the absence of ethylene

facilitate the ubiquitin-mediated proteasomal degradation of EIN3/EIL1 transcription factors (Fernandez-Moreno and Stepanova, 2020). EIN3 mediates ethylene signaling downstream of EIN2 (An et al., 2010). In the presence of ethylene, CTR1 is blocked by ethylene receptors which enables EIN2 to enhance ethylene responses by triggering the proteasomal degradation of EBF1/EBF2 (An et al., 2010).

TIR1 in auxin signaling

The phytohormone auxin is a major regulator of growth and development which controls a variety of diverse responses in plants (Woodward and Bartel, 2005). TRANSPORT INHIBITOR RESPONSE 1 and AUXIN SIGNALING F-box (TIR1/AFB) proteins serve as receptors, mediating a wide range of reactions in response to the plant hormone auxin (Dharmasiri et al., 2005; Parry et al., 2009). TIR1, a component of the ubiquitin-ligase (E3) complex SKP1-CUL-FBP (SCF), is the closest homologue to COI1 and its stability is enhanced when it assembles with the SCF complex (Yu et al., 2022; Yan et al., 2013). Auxin acts by facilitating an interaction between the Aux/IAA repressor proteins and the TIR1 subunit of the ubiquitin protein ligase SCF^{TIR1}. The Aux/IAA proteins are then ubiquitinated (Dos Santos Maraschin et al., 2009) and degraded by the 26S proteasome resulting in the release of inhibition of auxin response factors (ARFs). ARF proteins directly bind to DNA and can either activate or repress transcription depending on the ARF (Hagen and Guilfoyle, 2002). Members of the TIR1/AFB family are identified by a conserved F-box domain at their N-terminus, followed by 18 leucine-rich repeats. Upon auxin perception, TIR1/AFB proteins act as positive regulators of downstream auxin-responsive pathways (Du et al., 2022). The expansion of the Aux/IAA family probably resulted in a more efficient suppression of gene activity in the absence of auxin and a more precisely controlled auxin response system (Mutte et al., 2018).

TF phosphorylation

Proteins in general and TFs especially can undergo many posttranscriptional modifications that modulate protein activity. For example, histones can be acetylated, phosphorylated, methylated, sumoylated, ubiquinated, and ADP-ribosylated, and this is not an exhaustive list. In histones, these modifications are thought to form a code signifying the fate of the DNA, e.g. ready for transcription, repair, replication or condensation.

Phosphorylation is probably the best-studied posttranscriptional modification, certainly for transcription factors. The phosphate donor is usually ATP, and the phosphate group is mostly attached to serines or threonines, with a small percentage of tyrosine phosphorylation. In plants, as in microorganisms, the phosphate can also be transferred from one protein (domain) to another protein (domain) via a process called phosphorelay. In that case, also other aa can be phosphate acceptors such as aspartic acid and histidine. A well-known example of this process is in cytokinin signaling where the receptor in the plasma membrane first autophosphorylates via phosphorelay

and then transfers the phosphoryl group to a phosphotransfer protein from where it is transferred in the nucleus to a response regulator TF which thereby becomes active.
Phosphorylation can change transcription factor activity in many ways. It can affect stability, subcellular localization, interaction with positive or negative co-regulators, or affinity for its DNA target site and these are not mutually exclusive mechanisms.
Many classes of protein kinases using ATP as the phosphate donor are present in plants (Krupa et al., 2006). In *Arabidopsis*, disregarding the receptor kinase family which contains about half of the kinases found in the *Arabidopsis* genome, about nine families can be distinguished, the MAPK cascade kinases (MAPK, MKK, MKKK), casein kinases, and the AGC, cyclin-dependent, glycogen synthase, Raf, calcium-dependent, SNF1 and NimA kinases. A well-studied class of Ser/Thr kinases is formed by the mitogen-activated protein kinases (MAPKs), which are part of a MAPK cascade. There are many reports about TF phosphorylation by MAPKs. An example is ERF6 from *A. thaliana*, which is stabilized by phosphorylation by MPK3/MPK6, which has a stimulating effect on the defense response against fungal infection (Meng et al., 2013). Another superfamily of Ser/Thr protein kinases in eukaryotic cells are the casein kinases (CKs). The name is based on the fact that these kinases can phosphorylate the model substrate casein *in vitro*. There are two main subfamilies, casein kinase I (CKI) and casein kinase II (CKII). These are unrelated proteins, except for the fact that casein is an *in vitro* substrate and that phosphorylation occurs at Ser/Thr residues. In addition, there is G-CK, or genuine casein kinase, which has casein as a natural substrate that it phosphorylates in the Golgi apparatus of the lactating mammary gland. In terms of TF phosphorylation, CKII is the best-studied group. An example is the phosphorylation of the basic leucine zipper (bZIP) TF TGA5 in rice by CKII, which reduces its affinity for DNA target sites resulting in compromised expression of defense-related genes (Niu et al., 2022). The CK1 subfamily consists of two groups in plants, the casein 1-like (CKL) group with higher similarity to CKIs in other organisms, and a plant-specific group containing the Mut9p-LIKE KINASEs (MLKs) (Kang and Wang, 2020). The interest in CKI functions is increasing especially for the MLK group. MLKs have been shown to be involved in among others light signaling, circadian rhythms and phytohormone signaling (Kang and Wang, 2020).

Outline of this thesis

The goals of the thesis project were to investigate whether the super-MYC2 mutant CrMYC2^{D126N} can be used to enhance MIA biosynthesis in cell suspension (Chapter 2), and study the role of an ORCA-interacting F-box protein (Chapter 3) and the role of ORCA-interacting casein kinase I members (Chapter 4).
Chapter 2 describes experiments to determine the effect of CrMYC2 and CrMYC2^{D126N} (Super-MYC2) on the MIA biosynthesis and genes in the pathway. CrMYC2^{D126N} is a mutant that has no interaction with certain members of the CrJAZ family.

Chapter 3 describes studies about the role of F-box protein O2.51 in ORCA activity and MIA biosynthesis. It was found by interaction with ORCA2 in yeast two-hybrid screening. The co-expression of O2.51 in transient trans-activation assays in *C. roseus* cells negatively affected the activities of both ORCA2 and ORCA3. One of the goals of the research was to determine the effect of O2.51 on the MIA pathway in stably transformed overexpression and RNAi silencing cell lines.
Chapter 4 presents studies on the role of four casein kinase I (CKI) protein family members on ORCA activity and MIA biosynthesis. CKI members were found in yeast two-hybrid screening with ORCA3. The CKIs were able to phosphorylate ORCA2 and ORCA3 *in vitro*. The co-expression of CKI-1 in transient trans-activation assays in *C. roseus* cells negatively affected the activities of both ORCA2 and ORCA3. One of the research goals was to determine the effect of CKI on the MIA pathway in stably transformed overexpression cell lines.

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