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Darehei, M.

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Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in *Catharanthus roseus*

Mina Darehei
مینا دره ئی

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Promotores:
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Dr. S. Balazadeh

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Prof. dr. A.H. Meijer

Prof. dr. R. Offringa

Dr. S. Irmisch

Prof. dr. B. Mueller-Roeber, Potsdam University, Germany

Prof. dr. A. Goossens, Ghent University, Belgium

Dedicated wholeheartedly to B. A. A. (Aj), S. Ali and H. Ghasem

Contents

Chapter 1

General introduction

Mina Darehei and Johan Memelink

Plant cluster, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

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).

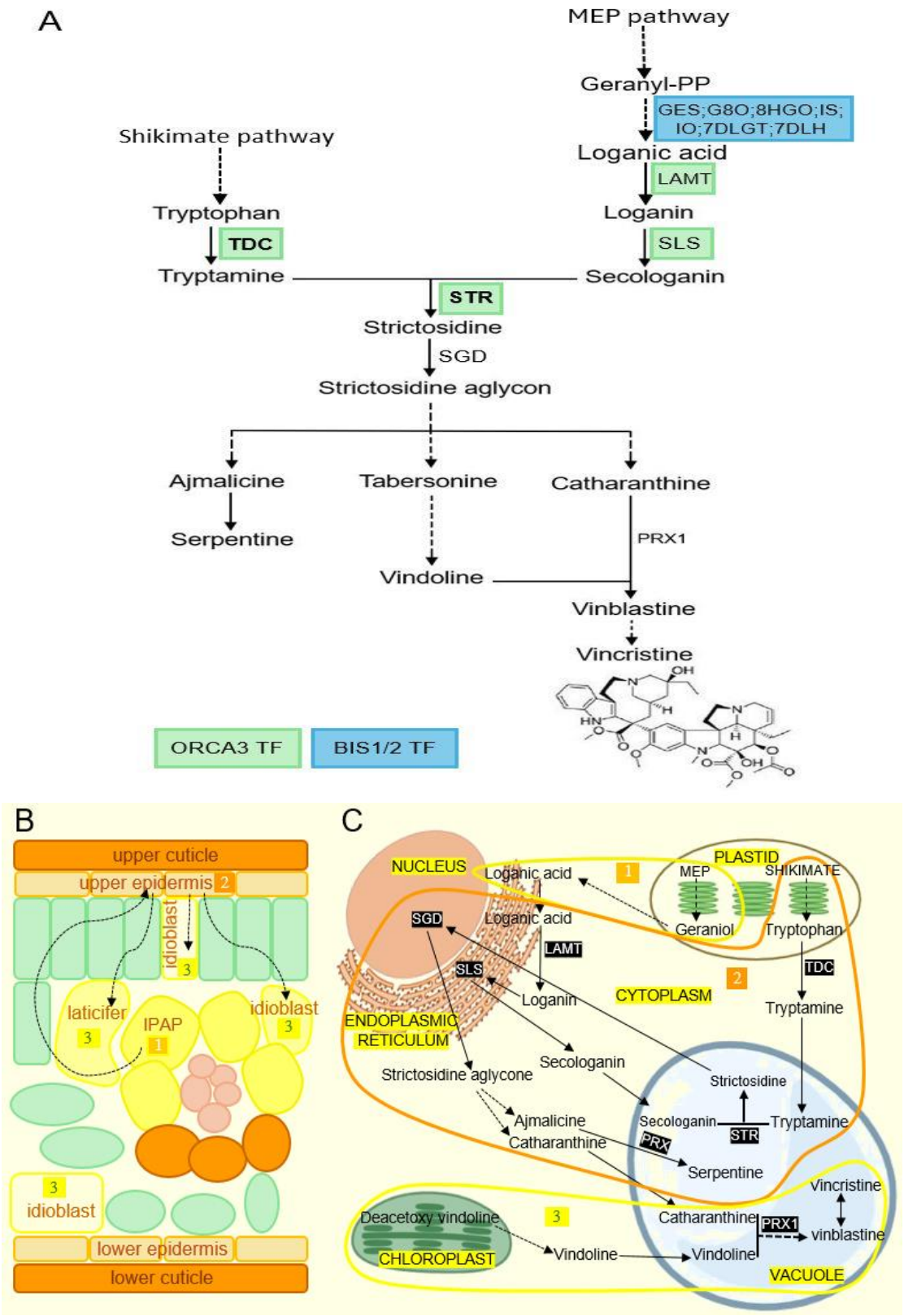

Figure 1. Schematic overview of the MIA pathway in *C. roseus* leading to the production of vinblastine and vincristine. (A) Pathway resulting in the production of shoot-specific MIAs in *C.roseus*. Genes regulated by BIS1 and ORCA are boxed in blue and green, respectively. (B) The MIA network and the 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.

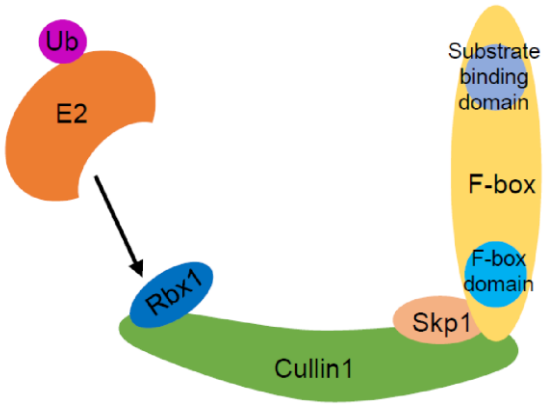

Figure 2. An SCF complex is composed of four primary subunits- Cullin1, Rbx1, Skp1, and an F-box protein. The Cullin1, Rbx1, and Skp1 subunits form the core ligase activity, with Rbx1 recruiting the E2 enzyme bearing an activated Ubiquitin molecule. F-box proteins play a vital role in delivering the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

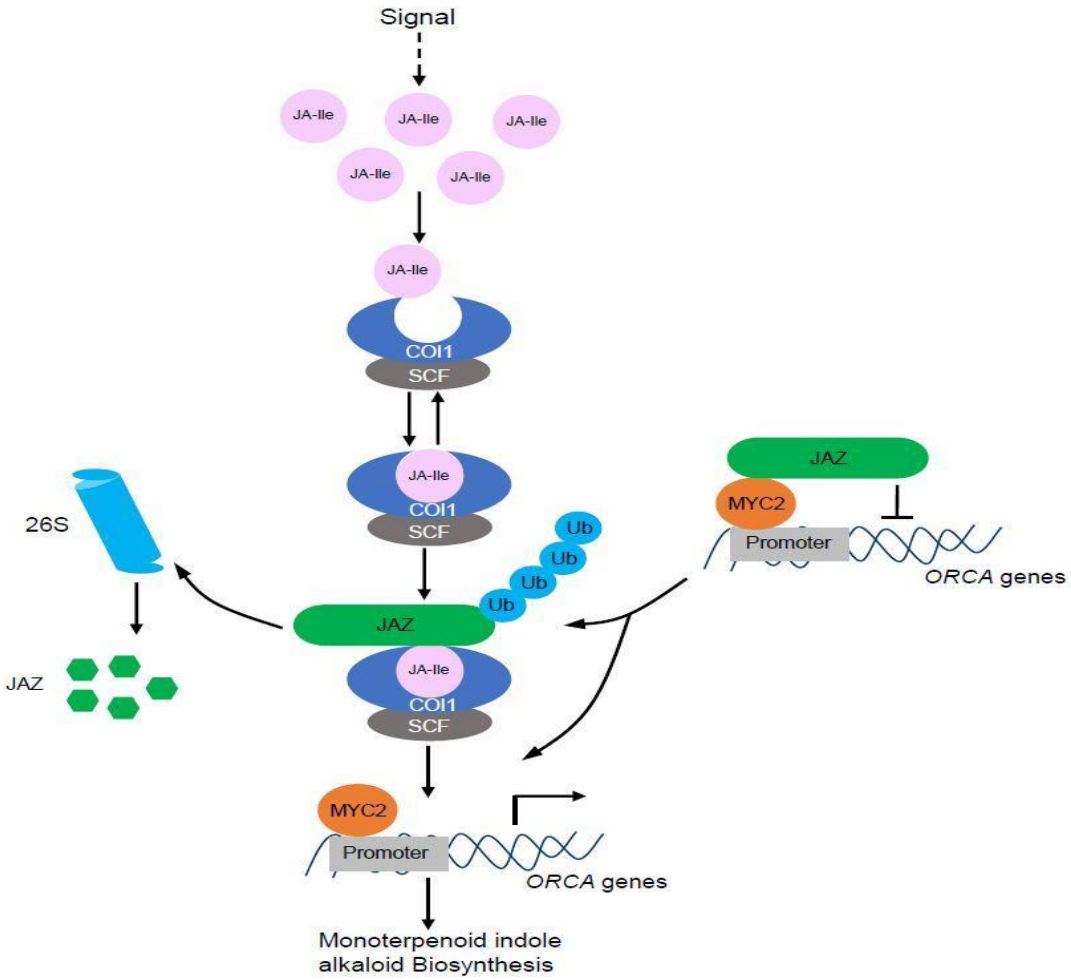

Figure 3. Model for JAs signal transduction leading to the expression of MIA biosynthesis genes in *C. roseus*. The F-box protein COI1 binds JA-Ile and successively recruits JAZ proteins for subsequent ubiquitination and degradation. JAZ repressors normally repress MYC2 activity among other TFs. Following 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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Chapter 2

Can engineered de-repressed CrMYC2a boost MIA biosynthesis in *C. roseus* suspension cells?

Mina Darehei*, Niki Savelkoel* and Johan Memelink*

Plant Cluster, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

* These authors equally contributed to the experiments.

Abstract

Catharanthus roseus is a medicinal plant that produces more than 200 monoterpenoid indole alkaloids (MIAs) including the valuable anticancer compounds vincristine and vinblastine. The content of these compounds is very low in the plant. To increase the production of these compounds, it is important to understand MIA pathway regulation. CrMYC2a is an important transcription factor regulating the MIA pathway but it is repressed by members of the JAZ family. JAZ proteins are degraded when jasmonates are produced, however, there is a negative feedback loop in which CrMYC2a activates the expression of *JAZ* genes. The research described in this chapter aimed at investigating the role of CrMYC2a with the use of overexpression cell lines of wild-type *CrMYC2a* and *CrMYC2a*^{D126N} (super MYC) and control lines expressing the *GUS* reporter gene. CrMYC2a^{D126N} is a mutant that cannot interact with certain members of the CrJAZ family. Overexpression constructs were based on an estradiol-inducible expression system or on the constitutive CaMV *35S* promoter. With the *35S* promoter, no *CrMYC2a* overexpression lines were obtained. With the inducible system, no overexpression lines for *CrMYC2a*^{D126N} were obtained, whereas three wild-type *CrMYC2a* and three *GUS* cell lines showed overexpression. Overexpressing wild-type CrMYC2a had a minor inducing effect on the *ORCA* genes and a strong inducing effect on the *CrJAZ1* gene. However, an inducing effect was not observed for genes that are direct targets of the ORCAs. In conclusion, we were unable to study whether it is possible to increase MIA production by CrMYC2a overexpression, due to the failure or maybe the impossibility of generating stable *CrMYC2a*^{D126N} overexpression lines.

Key words: *Catharanthus*, CrMYC2a, CrMYC2a^{D126N}, monoterpenoid indole alkaloids

Introduction

Catharanthus roseus with the common name Madagascar periwinkle is an important medicinal plant for the production of monoterpenoid indole alkaloids (MIAs), including the valuable anticancer drugs vincristine and vinblastine. However, the production of these compounds in *C. roseus* is very low, around 0,0002% of the fresh weight (Dinda et al., 2007), leading to high costs of vincristine and vinblastine. Due to their complex chemical structure, chemical synthesis is not an economically viable process. To enhance the production of MIAs in *C. roseus*, understanding the MIA pathway is crucial.

MIA biosynthesis is induced by jasmonates (JAs) (Zhou and Memelink, 2016; Memelink, 2009). Jasmonates constitute a family of bioactive oxylipins which are involved in several distinct processes in the plant. They play a role in the development of the plant but also have an important role in defense. When the plant is attacked by herbivores or pathogenic microorganisms, JAs are produced. In *C. roseus*, JAs induce the expression of transcription factor genes and biosynthesis genes in the MIA pathway, leading to increased MIA production.

MIAs contain two building blocks. The indole moiety is tryptamine derived from tryptophan, and the terpenoid moiety secologanin is derived from geraniol. The enzyme strictosidine synthase (STR) is responsible for the condensation of these two compounds, resulting in the synthesis of the monomeric alkaloid 3α(S)-Strictosidine. This can then be further converted via multiple steps into the dimeric alkaloids vincristine and vinblastine.

A region in the STR promoter can interact with two transcription factors (TFs) called ORCA2 (Octadecanoid derivative-Responsive Catharanthus AP2-domain) (Menke et al., 1999) and ORCA3 (Van der Fits and Memelink, 2000). The overexpression of ORCA2 and ORCA3 TFs cause elevated levels of expression of *STR* and several other MIA biosynthesis genes (Li et al., 2013; Van der Fits and Memelink, 2000).

ORCA gene expression is induced by JAs (Van der Fits and Memelink, 2001), which is regulated by the basic helix-loop-helix (bHLH) TF CrMYC2a (Zhang et al., 2011). Reduction of CrMYC2a expression by RNA interference blocked *ORCA* gene expression, demonstrating that CrMYC2a regulates JAs-responsive *ORCA* gene expression (Zhang et al., 2011).

CrMYC2a is regulated by the repressors Jasmonate ZIM-domain (JAZ) proteins (Zhang, 2008; Patra et al., 2018). Interaction between JAZ repressors and the MYC TFs is key in the JA signaling cascade (Goossens et al., 2015). The JAZ/MYC2 regulatory complex is widely conserved among the plant kingdom (Chini et al., 2016; Wasternack and Strnad, 2018). In general, in the absence of JA, in particular its bioactive form JA-isoleucine (JA-Ile), the activity of positive regulators of the JA response, such as the TF CrMYC2a, is blocked by the interaction with CrJAZ proteins (Schweizer et al., 2018; Patra et al., 2018). CrMYC2a TFs have a JAZ interacting domain (JID) (Goossens et al., 2015; Schweizer et al., 2018).

When JAs are produced, CrJAZ is degraded, therefore TFs such as CrMYC2a are de-repressed, leading to the transcriptional activation of their target genes (Schweizer et al., 2018; Zhang, 2008; Patra et al., 2018).
The *CrJAZ* genes repress the *CrMYC2a* gene, but the expression of Cr*JAZ* genes is also dependent on CrMYC2a. Knocking down *CrMYC2a* results in a reduction of *CrJAZ* gene expression (Zhang et al., 2011). This means there is a negative feedback regulation.
It has been shown that specific amino acid changes in the JID of *Arabidopsis thaliana* MYC2 (AtMYC2) can prevent the interaction with the certain JAZ proteins, resulting in a partially derepressed form, such as AtMYC2^{D105N} (Goossens et al., 2015). The negatively charged aspartate (D) at position 105 of AtMYC2 that causes the de-repression phenotype, corresponds to D126 of CrMYC2a (Schweizer et al., 2018). The CrMYC2a^{D126N} cannot interact with the CrJAZ3 and CrJAZ8 members of the CrJAZ family (Goossens et al., 2015; Schweizer et al., 2018). This mutant is useful for studying CrMYC2a function because overexpressing wild-type CrMYC2a has no effects as it also induces *CrJAZ* expression, which leads to *CrMYC2a* repression (Zhang, 2008). The use of the mutant CrMYC2a^{D126N} can therefore give more insight into the activity of CrMYC2a.
We attempted to generate stably transformed *C. roseus* lines constitutively or inducibly overexpressing *CrMYC2a, CrMYC2a^{D126N}* or *GUS* (control). Constitutive expression was based on the Cauliflower Mosaic Virus (CaMV) *35S* promoter. For inducible expression, an estradiol-inducible system was used which is based on the chimeric TF XVE, where E stands for the estradiol-binding domain of the human estrogen receptor. Estradiol causes migration of XVE to the nucleus resulting in expression of the XVE-controlled cassette containing the gene of interest (Zuo et al., 2000).
Due to the failure or maybe the impossibility of generating stable *CrMYC2a^{D126N}* overexpression lines even using the estradiol-inducible system, we were unable to study whether it is possible to increase MIA production by *CrMYC2a* overexpression.

Result

Identification of suitable *CrMYC2a* and *GUS* overexpression lines

The TF CrMYC2a is a master regulator in the JA signaling pathway. CrMYC2a interacts with the CrJAZ repressors and is maintained in a repressed state in the absence of JAs (Goossens et al., 2016). Recently it has been shown that specific amino acid changes in the JAZ interaction domain (JID) of CrMYC2a can prevent the interaction with certain members of the JAZ family (Schweizer et al., 2018), resulting in a de-repressed active form, such as CrMYC2a^{D126N} (Schweizer et al., 2018).
For constitutive overexpression of *CrMYC2a* and *CrMYC2a^{D126N}*, the genes were cloned in a *35S* expression cassette (Schweizer et al., 2018). For inducible overexpression the pER8 system was used (Zuo et al., 2000), which relies on the estradiol-responsive hybrid XVE transcription factor. XVE is a chimeric transcription factor containing the DNA-binding domain of the *Escherichia. coli* repressor Locus for

X-ray sensitivity A **(**LexA; X), the transcription activation domain of *Herpes simplex* viral protein 16 (VP16; V) and the hormone-binding region of the human estrogen receptor (E). A constitutive synthetic promoter controls XVE expression (Ishige et al., 1999). The XVE activator in transgenic plants is claimed to be tightly controlled by estradiol, and claimed to show no detectable transactivation activity without the inducer. Upon induction, the activator can enhance the expression of a reporter gene to levels more than eight times higher than those achieved with a *35S* promoter (Zuo et al., 2000).

C. roseus cells were transformed through particle co-bombardment with a hygromycin selection gene. Individual hyg^R calli were converted to cell suspensions, which were analysed for the expression of the introduced genes. Cell lines obtained by bombardment with the inducible constructs were treated with 10 µM estradiol for 24 hours before harvesting for RNA isolation.

Bombardment with the inducible wild-type *CrMYC2a* construct resulted in 28 stable cell lines, bombardment with the inducible *CrMYC2a*^{D126N} construct resulted in 14 stable cell lines and bombardment with the inducible *GUS* construct resulted in 25 stable cell lines. Bombardment with the *35S*-controlled *CrMYC2a* resulted in seven stable cell lines and with *35S*:*CrMYC2a*^{D126N} in five stable cell lines.

RNA blots with RNA from estradiol-inducible cell lines with *CrMYC2a* and *CrMYC2a*^{D126N} were analysed for overexpression of *MYC2*. In figure 1A is shown that out of the 28 cell lines, six showed higher RNA expression and cell lines #4, #6, #21, and #29 were kept for further investigation since we were aiming at having three replicate cell lines. Cell lines #3 and #8 also showed expression, but less than #4, #6 and #21. Cell line #29 was kept as an extra backup since it grew very well.

Of the 14 *CrMYC2a*^{D126N} cell lines (Fig. 1B) none showed a level of expression similar to the positive *CrMYC2a* or *GUS* cell lines. Cell line #14 showed the highest expression level. Despite their low level of *CrMYC2a*^{D126N} expression, cell lines #7, #10, #12 and #14 were kept for further analysis. Of the *GUS* cell lines, #2, #6 and #9 were kept, because they showed a high expression level of the *GUS* gene. As a control, all cell lines were also analysed for the expression of the household gene *RPS9* encoding small ribosomal subunit protein 9, which showed that lanes had similar RNA loading.

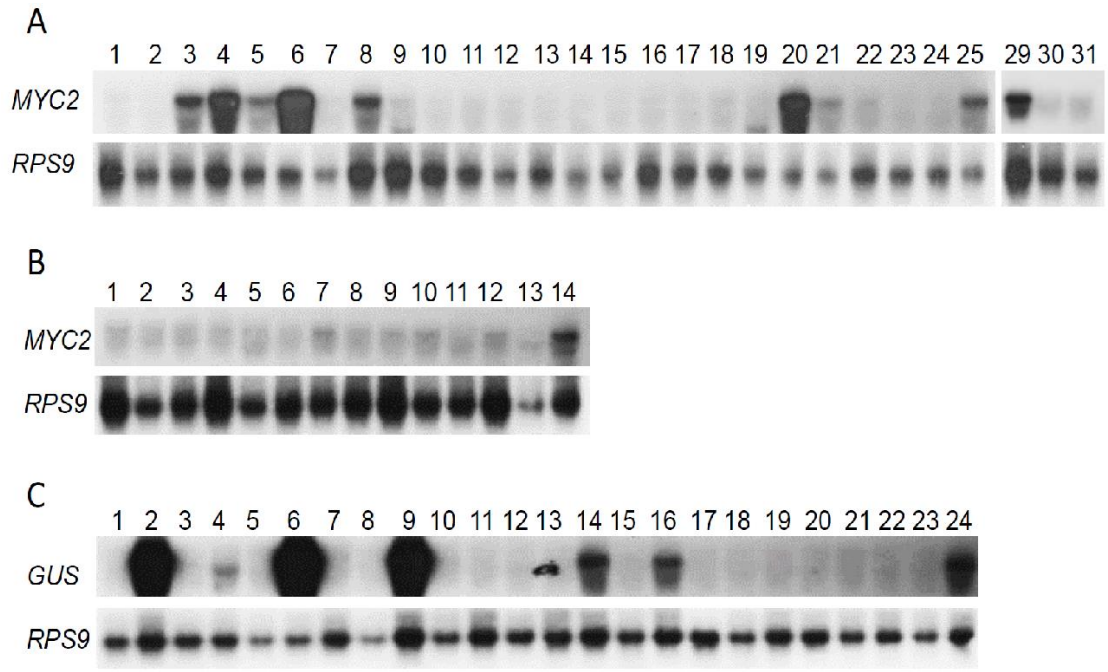

Figure 1. Analysis of transgene expression levels in stable hyg^R cell lines by RNA gel blot hybridisation. The generated cell lines were treated with 10 µM estradiol for 24 hours and analysed for overexpression of the bombarded genes. (A) Cell lines bombarded with wild-type *CrMYC2a* in plasmid pER8 (Zuo et al., 2000) were analysed for *CrMYC2a* overexpression. (B) Cell lines bombarded with the mutant *CrMYC2a*^{D126N} in pER8 were analysed for *CrMYC2a* overexpression. (C) Control cell lines bombarded with the *GUS* pER8 construct were analysed for *GUS* overexpression. Replicate blots were hybridized with radio-labeled *RPS9* encoding the small ribosomal subunit protein 9 as a loading control.

Analysis of *CrMYC2a* expression in cell lines bombarded with *35S* constructs

Cell lines were also generated with *CrMYC2a* derivatives under the control of the CaMV *35S* promoter. This is a strong constitutive promoter.

Ten stable cell lines were obtained after bombardment with the wild-type *CrMYC2a*. However, low expression of *CrMYC2a* was observed (Fig. 2). Cell lines #4, #5 and #9 were kept, showing the highest level of expression. With *CrMYC2a*^{D126N}, only five stable cell lines were obtained and cell lines #1, #4 and #5 were kept because a low level of *CrMYC2a* expression was observed (Fig. 2).

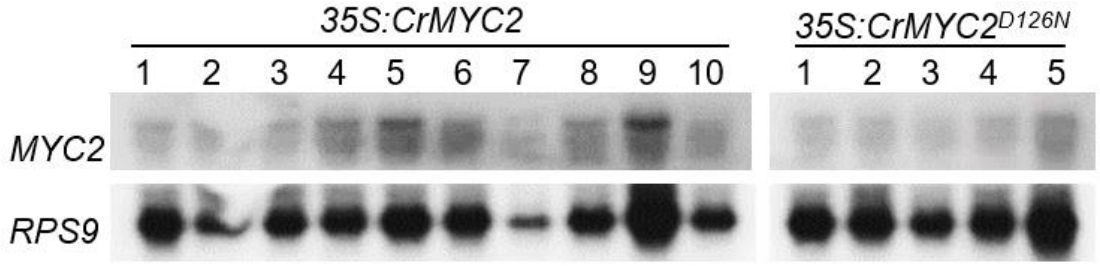

Figure 2. Analysis of the cell lines obtained by bombardment with *35S:CrMYC2a* and *35S:CrMYC2a*^{D126N}. RNA gel blots were hybridized with radio-labeled *CrMYC2a* or *RPS9* as a control.

Analysis of the expression of TF genes and biosynthesis genes in the MIA pathway

For the estradiol-inducible cell lines that showed overexpression, further research was done by looking at the expression of different genes that are reported as direct targets of CrMYC2a, and possible indirect targets. The cell lines were also analysed for the expression of the genes *G80* and *DXR*. This was done to see if there is an effect of CrMYC2a on other parts of the pathway of the production of MIAs. *G80* is active in the iridoid pathway leading to secologanin biosynthesis from geraniol (Collu et al., 2001) and *DXR* is active in the MEP pathway, which leads to the production of geraniol (Oudin et al., 2007).

The estradiol-inducible cell lines were treated for 24 hours with either 10 µM estradiol or an equivalent amount of the solvent DMSO. RNA gel blots were hybridized with different genes from the MIA pathway. The results are shown in figure 3. Cell lines expressing wild-type *CrMYC2a* showed a clear difference in *CrMYC2a* expression after treatment with estradiol compared to DMSO, indicating that the inducible overexpression worked. The expression of the CrMYC2a direct target genes *ORCA2* and *ORCA3* was not affected by the overexpression of *CrMYC2a* (Fig. 3). Only a weak possible effect was observed in cell line #29 for *ORCA3* expression. The *CrJAZ1* gene is also a direct target of CrMYC2a and estradiol treatment resulted in a higher level of Cr*JAZ1* expression. Overexpression of the wild-type *CrMYC2* did not affect *STR*, *TDC*, *G8O* or *DXR* expression (Fig. 3).

Cell lines obtained by bombardment with *CrMYC2a*^{D126N} showed low expression of *CrMYC2a* at levels similar to the *GUS* lines (Fig. 3). There was also not a big difference, if any, between the cells treated with estradiol and DMSO. Unsurprisingly, there was no effect of estradiol treatment on the expression of genes from the MIA pathway. However, in cell line #7, there was a small effect visible on the expression of the genes *G8O* and *DXR* and in cell line #14, there was an effect on the expression of the Cr*JAZ1* gene.

The *GUS* lines showed high levels of estradiol-inducible *GUS* expression. Estradiol treatment had no effect on the expression of any of the other genes analysed, which showed that estradiol itself or the estradiol-inducible XVE TF did not affect the expression of the tested genes and that the estradiol effects observed in the *CrMYC2a* lines are due to expression of wild-type or mutant *CrMYC2a* from the estradiol-inducible cassette.

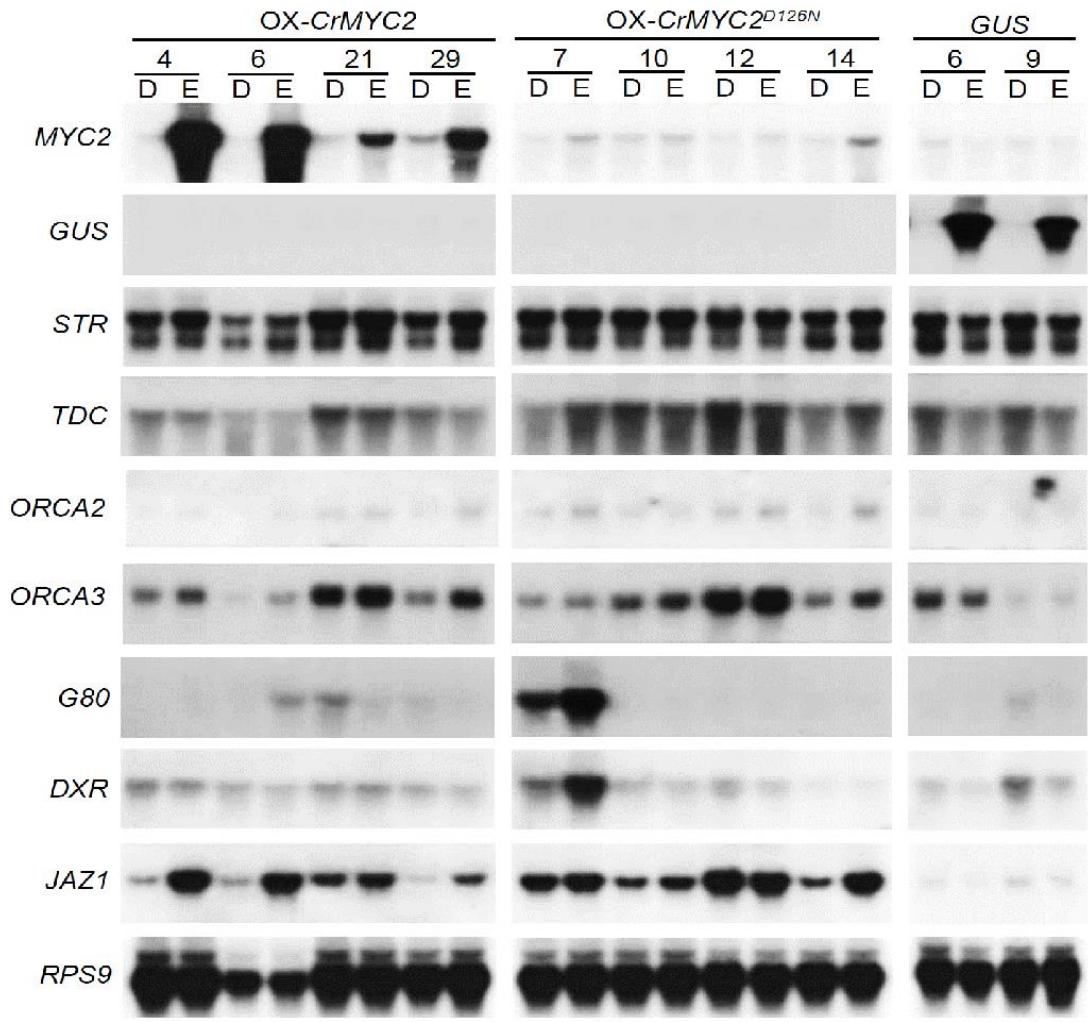

Figure 3. Analysis of the expression of TF genes and biosynthesis genes from the MIA pathway. Selected cell lines were treated with 0.1% DMSO (D) or 10 µM estradiol (E) for 24 hours. The extracted RNAs were analysed by RNA gel blot hybridization for the expression levels of different genes using *RPS9* as a control. Genes bombarded are shown on top, genes used as radio-labeled probes at the left. The three blots on a horizontal line were hybridized and exposed to film as one piece of blotting membrane. *ORCA2*, octadecanoid-derivative responsive *Catharanthus* AP2-domain protein 2; *ORCA3*, octadecanoid-derivative responsive *Catharanthus* AP2-domain protein 3; *G8O*, geraniol 8-oxidase; *TDC*, tryptophan decarboxylase; *STR*, strictosidine synthase; *DXR*, 1-deoxy-d-xylulose 5-phosphate reductoisomerase; *JAZ1*, jasmonate ZIM-domain protein 1; *GUS*, *E. coli* β-glucuronidase; *RPS9*, small ribosomal subunit protein 9.

Generation and analysis of a larger set of *CrMYC2a^{D126N}* cell lines

Since in the experiment described above, no overexpression of *CrMYC2a^{D126N}* was found, we generated a larger set of stable cell lines after bombardment with the inducible *CrMYC2a^{D126N}* construct. We also generated a new set of inducible *GUS* lines to verify that the whole procedure was working as expected. This time we were able to generate 30 stable cell lines for the *CrMYC2a^{D126N}* construct and 15 lines for the *GUS* construct. Cell lines were treated with 10 µM estradiol for 24 hours and the extracted RNA was screened for *CrMYC2* and *GUS* expression levels (Fig. 4). This showed that *CrMYC2a^{D126N}* lines #10, #11, #20, #24, #26, #27 and #28 had *CrMYC2a* expression levels that were low but somewhat higher than the other lines. For the *GUS* lines, #3,

#6, #9, #12 and #14 had detectable albeit variable *GUS* expression levels. The selected cell lines were then treated either with 0.1% DMSO (D) or 10 µM estradiol for 24 hours. The previously selected cell lines inducibly expressing wild-type *CrMYC2a* were taken along in the procedure as a control that the estradiol induction was working and as a reference level for *CrMYC2a* expression. Extracted RNAs were then analysed for expression of the bombarded genes by RNA gel blot hybridization. Three of the selected *GUS* lines had high levels of estradiol-inducible *GUS* expression (Fig. 5), showing that the procedure for generating cell lines with estradiol-inducible gene expression was working. The previously selected wild-type *CrMYC2a* lines showed estradiol-inducible *CrMYC2a* expression at similar levels as in the previous experiment, showing that the estradiol treatment was working and giving a reference level for reasonably high *CrMYC2a* expression. The *CrMYC2a*^{D126N} lines showed low *CrMYC2a* expression, and the detected *CrMYC2a* expression was not estradiol-responsive. Since these cell lines were no better than previously isolated *CrMYC2a*^{D126N} lines, further analysis of gene expression in these lines was not pursued.

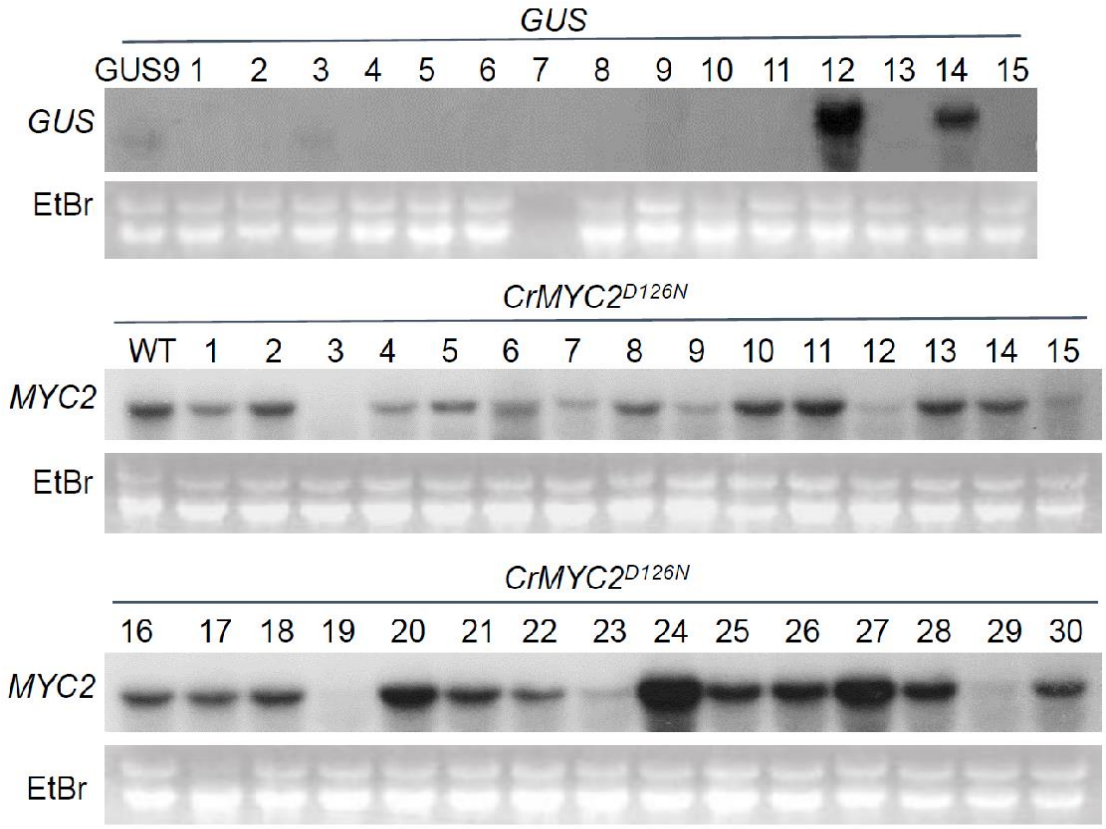

Figure 4. Expression analysis of *C. roseus* cell lines generated following particle bombardment with *CrMYC2a*^{D126N} in pER8. The generated cell lines were treated with 10 µM estradiol for 24 hours and analysed for overexpression of the bombarded genes. Northern blots containing identical amounts of total RNA from each line were hybridized with the *CrMYC2a* cDNA probe. Control cell lines were hybridized with *GUS* probe. The Ethidium Bromide (EtBr) stained gel is shown as a loading control. WT: Wild-type, GUS9: an old cell line that used to be positive.

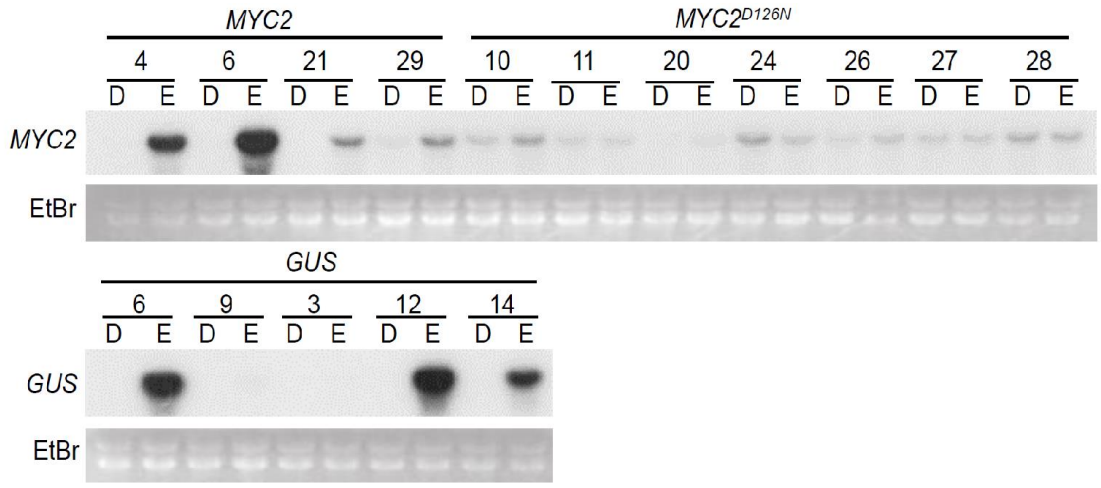

Figure 5. Analysis of transgene expression levels in stable hyg^R cell lines. Genes were in plasmid pER8. The cell lines were treated either with 10 µM estradiol (E) or 0.1% DMSO (D) for 24 hours and analysed for transgene expression levels by RNA gel blot hybridization. Genes bombarded are shown on top, genes used as radio-labelled probes at the left. The Ethidium Bromide (EtBr) stained gel is shown as a loading control.

Discussion

MIAs in *C. roseus* are economically important as they include the valuable compounds vincristine and vinblastine. Their levels in plants are low resulting in high prices for these effective cancer therapeutics. Increasing their levels in plants requires a thorough understanding of MIA biosynthesis and its regulation. From studies on the regulation of the MIA pathway, CrMYC2a emerged as a possible master regulator of the pathway (Goossens et al., 2015; Schweizer et al., 2018). However, a simple approach of overexpressing CrMYC2a is unlikely to be effective, because high *CrMYC2a* expression likely has toxic effects and because of the existence of a negative feedback loop wherein CrMYC2a stimulates the expression of its own repressors, the CrJAZ proteins. To circumvent these obstacles, for the research described in this chapter an approach was chosen with inducible *CrMYC2a* expression combined with the use of a *CrMYC2a* mutant which cannot interact anymore with several members of the CrJAZ family. To robustly validate the effects of an overexpressed gene, two to three independent replicate cell lines with clear overexpression are needed. For the wild-type CrMYC2a and the GUS control it was possible to obtain at least three cell lines that showed overexpression. The cell lines generated with *CrMYC2a*^{D126N} did not show the desired overexpression.

Previous reports (Schweizer et al., 2018) suggested that the use of *35S* as a constitutively active promoter could induce overexpression using the transient expression system based on agro-infiltration. However, we found that the constitutively active *35S* promoter did not lead to stably transformed cell lines showing overexpression of wild-type *CrMYC2a* or mutant *CrMYC2a*^{D126N}. The low total amount of hyg^R cell lines generated indicates that constitutive *CrMYC2a* overexpression has toxic effects.

With the inducible system, it was possible to obtain cell lines with overexpression of wild-type *CrMYC2a*. However, there were no or very minor effects on expression of *ORCA* genes which are direct targets of CrMYC2a. Also, the expression of *STR* and *TDC*, which are direct target genes of the ORCAs, was not affected. There was however increased expression of *CrJAZ1*. Thus, the explanation for the lack of effect of *CrMYC2a* overexpression is probably that *CrMYC2a* expression increases the expression of Cr*JAZ* genes, leading to *CrMYC2a* repression.
With the mutant *CrMYC2a*^{D126N}, it was not possible to obtain cell lines with inducible overexpression. The most likely explanation for this is in our view that the XVE expression system is a bit leaky, causing severe toxic effects with this powerful mutant *CrMYC2a* variant. Evidence for leakiness is the observation that Cr*JAZ1* has elevated expression in the *CrMYC2a*^{D126N} lines without estradiol treatment. Also, a close look at the *GUS* panel in figure 3, which is a bit overexposed, shows slight *GUS* expression in the DMSO-treated cell lines.
Thus finally, we could not study whether it is possible to increase MIA production by *CrMYC2a* overexpression, due to the failure or maybe the impossibility of generating stable overexpression lines. For scientific studies, it might be possible to use a transient expression based on agro-infiltration, but it is unlikely that this will make a good commercial production system. It might be possible to reduce the leakiness of inducible *CrMYC2a* expression by using a double inducible system, combining for example a dexamethasone-inducible *CrMYC2a*-glucocorticoid receptor fusion with the estradiol-inducible XVE system. In that case, the toxicity of *CrMYC2a-GR* expressed due to leakiness of the XVE system might be prevented by retention in the cytoplasm in the absence of dexamethasone.

Materials and methods

Plasmid constructs

The constructs for the cell lines with *35S:CrMYC2a* and *35S:CrMYC2a*^{D126N} were made by Schweizer et al. (2018) with the use of Gateway technology. The vector *pH7WG2D* was used for the plasmids *CrMYC2a* + stop and *CrMYC2a*^{D126N} + stop. No control was provided so cell lines without expression were planned to serve as control lines.
The *CrMYC2a* genes were amplified on the *35S*-constructs of Schweizer with the primers CTC GAG ATG ACG GAC TAT AGG CTA CAA C and CTC GAG TCT AGA TCA TAC CAA GAG CCT CAT CGA G. They were cut with Xhol and Xbal and cloned in pER8 digested with Xhol and Spel.

Cell culture, stable transformation, treatments

C. roseus cell suspension line MP183L was maintained by weekly 5-fold dilution in 20 ml of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 25˚C in a 16/8 hour light/dark regime at 200 µE m^{-2} S^{-1} at 60% relative humidity on a rotary shaker at 120 rpm. For stable transformation of cell line MP183L, the plasmid construct of interest was co-transformed with the

plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV *35S* promoter in a ratio of 4 to 1 by particle bombardment of a thin cell layer on a paper filter (Van der Fits and Memelink, 1997). Previous experience has learned that co-transformation efficiency is 10-15%. Paper filters with bombarded cells were transferred to a solid LS medium containing 50 µg/mL hygromycin-B and individual hygromycin-resistant calli emerging after about one month were transferred to the same medium and after about 1 month of further growth were converted to cell suspensions. Ten ml aliquots of the cell lines were incubated for 24 hours with either 10 µl DMSO (D) (0.1% final concentration) or 10 µl of 10 mM estradiol dissolved in DMSO (E) resulting in a 10 µM final estradiol concentration. Cells were then harvested by vacuum filtration on an 80 µM plastic filter, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80°C.

RNA isolation and Northern blot analysis

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose, 1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming (Decalabel DNA labeling kit, Thermo Scientific). (Pre-) hybridization and subsequent washing of blots were performed as described (Memelink et al., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *G8O* (AJ251269), *DXR* (AF250235), *GUS* (U02441), *STR* (X61932), *TDC* (M25151), *RPS9* (AJ749993), *CrMYC2a* (AF283507), *CrJAZ1* (FJ040204).

Isolation of plasmids and probes

Plasmid DNA for the bombardment was isolated with the QIAGEN plasmid Midikit 100® according to the manufacturer's instructions. Plasmids for analysis during cloning procedures were isolated using a laboratory protocol modified from Birnboim and Doly (1979). The probes *ORCA2* and *ORCA3* were amplified with PCR and afterwards isolated with a Gel Purification Kit (Thermo Scientific). PCR was done with a Thermo Scientific Phusion High-Fidelity DNA Polymerase with M13 forward and M13 reverse primers.

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Chapter 3

The role of F-box protein O2.51 in the regulation of alkaloid biosynthesis genes in *Catharanthus roseus*

Mina Darehei, Debora Vom Endt, Alessandra Biancucci, Angga Dwituti Lestari and Johan Memelink

Plant cluster, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

Abstract

Plants produce a large variety of secondary metabolites and many commercial pharmaceutical drugs are derived from these metabolites. The medicinal plant *Catharanthus roseus* produces two important monoterpenoid indole alkaloids (MIA) vincristine and vinblastine which are widely used for cancer treatment. Some genes in the MIA pathway including *strictosidine synthase* (*STR*) and *Tryptophan decarboxylase* (*TDC*), are regulated by the transcription factors Octadecanoid derivative-Responsive *Catharanthus* AP2-domain protein 2 and 3 (ORCA2 and ORCA3). Yeast two-hybrid screening identified a protein named O2.51 which interacted with ORCA2. It showed high similarity to *A. thaliana* F-box proteins EBF1/2, known for their interaction with the ethylene-responsive transcription factor ETHYLENE-INSENSITIVE 3 (EIN3) and the related EIL3. The F-box protein O2.51 could potentially function similarly in JA signaling, by interacting with ORCA2 and, probably, ORCA3. A hypothesis is that O2.51 may promote the degradation of ORCA2 and ORCA3 through the ubiquitin-26S proteasome system. The experiments detailed in this chapter aimed to explore the potential impact of O2.51 on ORCA activity and, subsequently, on MIA biosynthesis genes including *STR* and *TDC*. O2.51 interacted only with ORCA2 in yeast. However, the co-expression of O2.51 in transient trans-activation assays in *C. roseus* cells resulted in a negative impact on the activities of both ORCA2 and ORCA3. To investigate the effect of O2.51 on MIA biosynthesis genes, stable cell lines overexpressing *O2.51* or with silenced expression levels were generated. However, the number of replicate lines was too small and/or the gene expression was too variable to draw firm conclusions.

Keywords: *Catharanthus roseus*, MeJA, F-box protein, ORCA2, ORCA3

Introduction

Catharanthus roseus is one of the most studied medicinal plants because of the wide range of medicinal compounds it produces. *C. roseus* produces more than 130 monoterpenoid indole alkaloids (MIA), including vincristine and vinblastine. These compounds are commonly used in treating cancer (Hemmati et al., 2020). They are produced via the MIA pathway starting with the condensation of the indole moiety tryptamine with the monoterpene-seco-iridoid moiety secologanin (Oudin et al., 2007). This reaction is catalyzed by strictosidine synthase (STR).

Recently, there has been considerable progress in understanding the biosynthesis and the regulation of the MIA pathway. However, several regulatory steps of this pathway are still not well understood (Miettinen et al., 2014; Van Moerkercke et al., 2015; 2016). MIA biosynthesis is induced by the group of jasmonate plant hormones (JAs) (Memelink et al, 2001). JAs regulate plant responses to environmental and developmental cues (Memelink, 2009), such as the induction of secondary metabolites. (+)-7-iso-JA-L-Ile ((+)-7-iso-JA-Ile, JA-Ile), the endogenous active JAs molecule and coronatine (COR), a Pseudomonas syringae-produced phytotoxin that mimics JA-Ile, are able to efficiently trigger JA signaling (Yan et al., 2018). The MYC protein family, which are key regulators in JA signaling, are central TFs of the JA core signaling complex that interact with the Jasmonate ZIM Domain (JAZ) repressors and are maintained in a repressed state in the absence of JAs (Goossens et al., 2016). JA-Ile promotes the interaction between JAZs and the F-box protein Coronatine Insensitive1 (COI1). JAZs are ubiquitinated and degraded by the 26S proteasome, leading to the release of MYC2 and other downstream regulators and the transcriptional activation of target genes. The AP2/ERF transcription factors (TFs), Octadecanoid-Responsive Catharanthus AP2-domain protein 2 (ORCA2) and ORCA3 are major regulators of several MIA pathway genes. CrMYC2 has been shown to act upstream of ORCA2 and ORCA3, and to directly activate their transcription (Zhang et al., 2011). ORCA2 and ORCA3 subsequently induce the expression of several MIA genes including *STR*.

Here we used yeast two-hybrid screening to isolate proteins that interact with ORCA2, resulting in the F-box protein O2.51. This suggests that O2.51 is involved in the degradation of ORCA2. Although O2.51 interacted only with ORCA2 in yeast, it had a negative effect on the trans-activation activity of both ORCA2 and ORCA3. Another aim of this study was to generate stable transgenic cell lines with elevated or reduced expression of *O2.51* to study the effect on MIA biosynthesis.

Result

Yeast two-hybrid screening for ORCA2-interacting proteins

Since the full-length ORCA2 sequence resulted in strong auto-activation in yeast, the truncated version Δ5ORCA2 lacking an N-terminal acidic domain and without auto-activation activity was cloned in the yeast expression vector pAS2-1 creating a fusion protein between the truncated ORCA2 and the GAL4 DNA-binding domain. This construct was used to screen a cDNA library of elicitor-treated *C. roseus* suspension

cells in the yeast expression vector pACTII in yeast strain PJ69-4A. cDNA-encoded proteins are expressed as fusions with the GAL4 activation domain. PJ69-4A contains two nutritional markers (*HIS3* and *ADE2*) and a reporter gene (*MEL1)* driven by the GAL4-controlled *GAL1*, *GAL2* and *MEL1* promoters respectively (James et al., 1996). The use of different promoter-reporter genes eliminates false positives. The number of positive yeast transformants in each step of the screening is listed in Table 1. In the first round of screening, the transformed cells were plated on a medium lacking histidine. Colonies from these plates were then re-streaked on a medium lacking adenine. Growing yeast cells were then patched on plates containing X-α-Gal to detect α-galactosidase activity conferred by the GAL4-controlled *MEL1* gene. Plasmids were extracted from yeast colonies that turned blue on the last medium as a consequence of *MEL1* gene activation and transformed to *E.coli* for plasmid preparation. pACTII plasmids containing different cDNA inserts were then re-transformed to PJ69-4A together with the empty pAS2-1 plasmid, or with pAS2-1 containing Δ5ORCA2. Growth was compared and pACTII clones, which were able to confer growth only in the presence of the bait, were considered true positives (Table 1). Assuming that the mRNA population of *C. roseus* suspension-cultured cells has the standard complexity found in cells of higher eukaryotes, screening of 0.5 to 1.0 x 10^6 independent transformants with a unidirectional cDNA library results in a probability of $\geq$ 99% of screening every mRNA species (Klickstein, 1992). Thus, assuming that the cDNA library forms a faithful representation of the mRNA template, the yeast two-hybrid screening represented a near-complete screening of the mRNA population of elicitor-treated *C. roseus* cells (Table 1). Based on Southern blot cross-hybridization experiments positive clones were grouped in classes (Table 2). Unique clones and the longest clone from each cross-hybridization class were further characterized by sequencing, and sequence comparison with the NCBI DNA database (http://www.ncbi.nlm.nih.gov). A number of clones coding for proteins interacting with Δ5ORCA2 had homology to putative proteins in the database, which had no homology to other described proteins and are referred to as unknown proteins (Table 2).

Table 1. Number of yeast transformants screened using the yeast two-hybrid system and the number of positive clones that were isolated in each step of the screening with the Δ5ORCA2 bait.

Bait	**Δ5ORCA2**
Transformants screened	6.5 x 10^5
Histidine selection	448
Adenine selection	180
X-α-gal screening	159
Retransformation	19

Table 2. Classification of the positive clones found using Δ5ORCA2 as bait in yeast two-hybrid screening. Classes of cross-hybridizing clones are indicated. The column "# clones" represents the number of members of each class. For some classes, homology to proteins in the NCBI database is indicated. "Unknown protein" means that searches in NCBI database revealed homology to predicted proteins with no known function.

Classes	# clones	Homologous to
I	1	tRNA pseudouridine synthase
II	3	Glutathione S-transferase
III	3	Polyphenol Oxidase
IV	1	Unknown protein
V	1	Unknown protein
VI	7	Aspartic Proteinase
VII	1	Unknown Protein
VIII	1	F-box protein
IX	1	Pyruvate Kinase

Clone with homology to F-box proteins

Class O2.VIII was composed of one unique clone, numbered O2.51, which had similarity to a class of plant proteins bearing an N-terminal 60 amino acid F-box, followed by a variable number of Leu-rich repeats. The F-box interacts with the Skp1 component of the SCF (Skp1-Cullin-F-box) complex, whereas the Leu-rich repeats interact with the target protein, thereby targeting it for ubiquitination and degradation by the 26S proteasome. Clone O2.51 encoded only the Leu-rich repeat region. A full-length sequence, termed FL-O2.51, was retrieved from the ORCAE database (bioinformatics.psb.ugent.be/orcae; Van Moerkercke et al., 2013). The best *Arabidopsis* homologues of this *Catharanthus* F-box protein are the F-box proteins EBF1 and EBF2 (Fig. 1A). These two functionally redundant proteins target the transcription factors EIN3 and EIL1, key regulators of ethylene signaling, for degradation (Potuschak et al., 2003; Guo and Ecker, 2003; Gagne et al., 2004). Ethylene inhibits EBF-mediated EIN3 degradation resulting in nuclear accumulation of EIN3 protein which then activates ethylene-responsive genes (Fig. 1B). The ORCA2-interacting F-box protein FL-O2.51 could function in a similar way to regulate ORCA2 protein abundance, possibly in a JA-responsive manner.

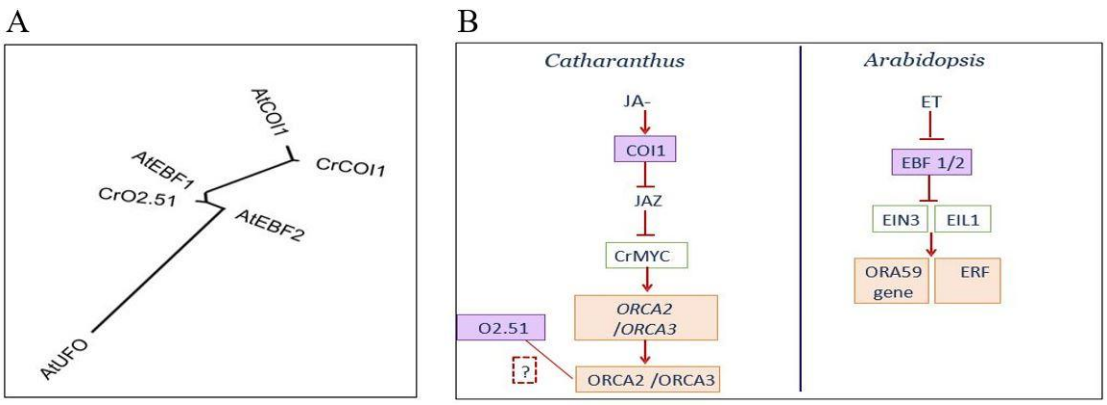

Figure 1. Phylogenetic relationship of related F-box proteins in *C. roseus* and *A. thaliana*. (A) Protein sequences of AtCOI1, CrCOI1, AtEBF1, AtEBF2, CrO2.51, and AtUFO were obtained from TAIR and ORCAE. They were aligned by using the Clustal W program and an unrooted phylogenetic tree was drawn by the neighbour-joining method. (B) Schematic representation of analogies between JA and ET hormonal signaling cascades. ET inhibits the ubiquitination of EIN3 via the $SCF^{EBF1/EBF2}$ complex, resulting in nuclear accumulation of the TFs EIN3 and EIL1. EIN3 and EIL1 activate genes encoding the TFs *ORA59* and several ERFs. JA results in degradation of JAZ repressors via the SCF^{COI1} complex resulting in the release of CrMYC2 from repression. CrMYC2 activates genes encoding TFs such as *ORCA2* and *ORCA3*. Purple boxed abbreviations indicate F-box proteins. Arrows depict activation, T-bars indicate inhibition, dashed line represents that the interaction still needs to be investigated.

FL-O2.51 interacts with Δ5ORCA2

The truncated clone O2.51 was found by interaction with Δ5ORCA2 and did not interact with Δ5ORCA3 (not shown). The interaction of FL-O2.51 with ORCA2 and ORCA3 was tested in yeast. FL-O2.51 was expressed as a fusion with the GAL4 activation domain from the plasmid pACTII. Deletion derivatives of ORCA2 and ORCA3, lacking the acidic activation domains, were expressed as fusions with the GAL4 DNA-binding domain (GAL4-BD) from yeast/*E.coli* shuttle vector pAS2-1. Six combinations of plasmids were tested in the yeast two-hybrid system. The BD and AD domains are brought together when the interaction between the bait protein and the prey protein occurs. This interaction activates the reporter genes and their transcription in yeast was evaluated by monitoring growth on the plates. All growth media lacked tryptophan and leucine (SD-LW), and depending on the selection, additionally lacked adenine (SD-LWA) or histidine and in the latter case also contained 3 amino-1,2,4-triazole (SD-LWH with 5 mM or 10 mM 3-AT). On non-selective growth media used as control, each combination of plasmids resulted in the growth of yeast transformants. FL-O2.51 interacted only with Δ5ORCA2 as evidenced by growth on the SD-LWA selection medium (Fig. 2). Yeast cells did not grow with combinations containing the empty vectors pAS2.1 or pACTII in any selection medium except for the non-selective control.

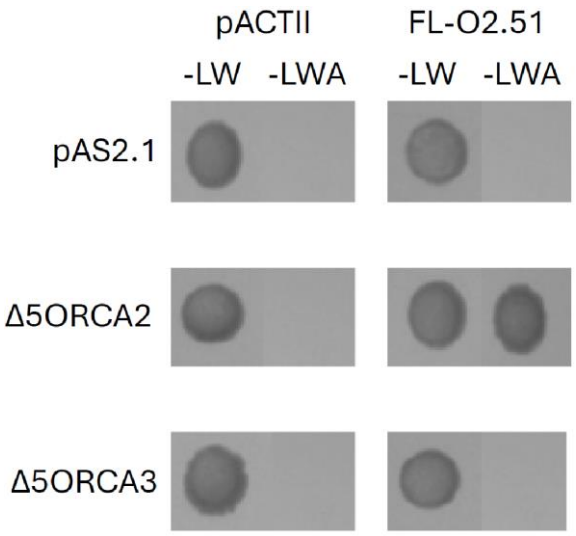

Figure 2. ORCA2 interacts with full-length O2.51. On the left side of each panel, yeast growth on a plate containing Synthetic Minimal Media (SD) lacking Leu, Trp (-LW). On the right side, yeast growth on plates containing SD medium lacking Leu, Trp, and Ade (SD-LWA) media. FL-O2.51 was cloned in pACTII, Δ5ORCA2 and Δ5ORCA3 were cloned in pAS2.1.

Generating *C. roseus* cell suspension lines overexpressing *O2.51*

C. roseus cell suspension lines overexpressing *O2.51* were generated using a construct with the full-length *O2.51* open reading frame fused to the CaMV *35S* promoter in the plasmid pRT101. The empty vector pRT101 was used to generate control cell lines. The *O2.51* overexpression construct and the empty control plasmid were introduced in cell line MP183L by particle bombardment together with a plasmid carrying a hygromycin resistance gene. The experience in the research group is that co-transformation frequency is in the order of 10-30 %. Around one month after the bombardment independent hygromycin-resistant calli were transferred onto new hygromycin selection plates. The calli were grown within around four weeks until a size of around 1-2 cm in diameter upon which they were transferred into liquid medium. After growth of the calli in the liquid medium was established after 2-3 weeks, the weekly transfer of the cell lines was started until stably growing cell lines were obtained (Fig. S1).

This bombardment experiment was relatively unsuccessful because we did not obtain any control suspension culture and only seven lines from the bombardment with *O2.51*. These lines were analysed for *O2.51* gene expression by Northern blot hybridization to select lines that effectively overexpress *O2.51* (Fig. 3). As a control for mRNA loading, the blot was probed with the *RPS9* gene encoding protein 9 of the small subunit of the cytoplasmic ribosome.

Out of the seven independent cell lines, two showed high *O2.51* mRNA levels, one line had a lower but elevated mRNA level, and four lines did not express *O2.51* at a measurable level. Lines 1 and 4 were kept as overexpression lines and lines 3 and 8 as control lines for further experiments.

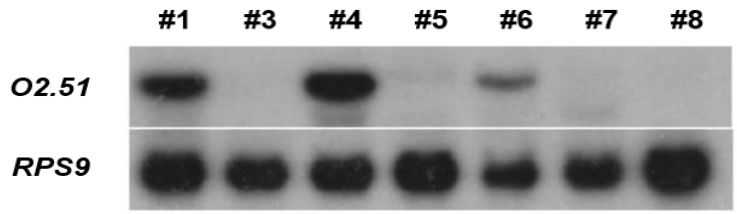

Figure 3. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* overexpression construct. Northern blots containing identical amounts of total RNA from each line were hybridized with the *O2.51* and *RPS9* cDNA probes. *RPS9* encodes the ribosomal protein 9 of the cytoplasmic small ribosomal subunit and was used as a loading control.

Effect of *O2.51* overexpression on expression of MIA biosynthesis genes

Using transgenic lines that constitutively overexpress the *O2.51* gene from the CaMV *35S* promoter, the role of O2.51 in MIA biosynthesis was assessed by measuring the mRNA levels of *STR* and *TDC*, since these two MIA biosynthesis genes are known to be regulated by ORCA2 and ORCA3 (Van der Fits and Memelink, 2000, Peebles et al., 2009; Li et al., 2013).

At 30 min of MeJA treatment no effect was observed on the expression of *TDC* and *STR* (Fig. 4) as reported previously (Van der Fits and Memelink, 2001). At 6 hrs of MeJA treatment increased expression of the *TDC* and *STR* genes was observed, whereas no effect was found on the expression of the control gene *RPS9*. In the two independent *O2.51* overexpression lines, the mRNA levels of *TDC* and *STR* were very similar between the two lines. In the control lines, however, the mRNA levels were quite different. In control line 3, high *TDC* and *STR* mRNA levels were observed without MeJA treatment, whereas in control line 8 without MeJA treatment the *TDC* and *STR* mRNA levels were extremely low. This difference in the expression of control cell lines was unexpected. The interpretation of the effect of *O2.51* overexpression on the expression of MIA biosynthesis genes was hindered by this high level of variability in the control lines.

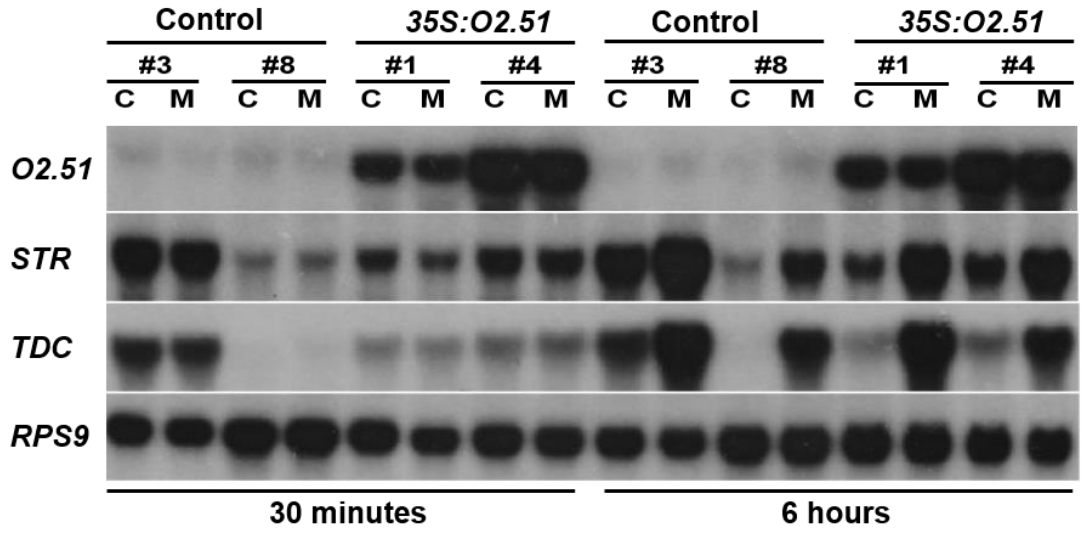

Figure 4. Effect of *O2.51* overexpression on MeJA-responsive expression of MIA biosynthesis genes. Two independent overexpression lines and two independent control lines were treated for 30 minutes and or 6 hours with 0.1% (v/v) DMSO and or with 10 µM MeJA/0.1% DMSO. Northern blots were hybridized with the *O2.51*, *STR*, *TDC* and *RPS9* cDNA probes. *RPS9* encodes the ribosomal protein 9 of the cytoplasmic small ribosomal subunit and was used as a loading control.

O2.51 gene expression is not induced by MeJA

Genes encoding the transcription factors ORCA2 and ORCA3 and MIA biosynthesis genes, including *TDC* and *STR* are induced by MeJA (Van der Fits and Memelink, 2001; Peebles et al., 2009; Li et al., 2013). Whether the gene *O2.51* responds to MeJA can be seen in the previous experiment (Fig. 4) by looking at the results for control lines 3 and 8. A detectable *O2.51* RNA band is observed, but the intensity does not change with longer MeJA treatment which showed that the *O2.51* gene was not induced by MeJA.

A new attempt to study the effect of *O2.51* overexpression on the expression of MIA biosynthesis genes

Given the high variability in the control lines and the low number of overexpression lines in the previous experiment, we made a new attempt to generate more lines.
In total, 92 transgenic cell lines were made from the bombardment with pRT101-*O2.51* and 8 lines were made with the empty vector. These lines were analysed for *O2.51* gene expression by Northern blot hybridization to select lines that effectively overexpress *O2.51*. Lines 3, 20, 32, 43, 64 and, 72 showed different expression patterns compared to controls and many lines did not express *O2.51* at a measurable level (Fig. S2). For confirmation of overexpression, a Reverse Transcriptase (RT) PCR was done. The RT products were then PCR amplified. The forward primer was designed based on the first 20 base pairs of the *O2.51* gene and the reverse primer was designed based on the CaMV *35S* transcriptional terminator of the pRT-101 plasmid. The PCR result showed that all cell lines express *O2.51* in comparison with controls (Fig. S3).

Overexpression cell lines together with 2 control cell lines were treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 3 time points (0, 0.5, 6 h). Northern blots were hybridized with *O2.51*, *ORCA2*, *ORCA3*, *STR*, and *TDC* probes, with *RPS9* as a loading control (Fig. S4). All O2.51 lines except 72 overexpressed smaller RNAs that were too short for the full-length *O2.51* mRNA. Line 72 overexpressed a large RNA that could be the full-length *O2.51*. *ORCA3* gene expression was rapidly and transiently induced by MeJA with high expression level as early as 30 min after MeJA addition, in both overexpression and control cell lines. *ORCA2* was induced at the 6 hr timepoint but showed variable expression between lines. *TDC* and *STR* mRNA levels were relatively consistent with the highest expression at the 6 hr timepoint. However, there were no consistent differences between overexpression and control lines.

Attempt to study the effect of *O2.51* silencing on the expression of MIA biosynthesis genes

To determine whether decreased levels of *O2.51* expression affect the expression of ORCA target genes, we tried to knock down *O2.51* expression via RNA interference (RNAi). The silencing *O2.51* construct was made by selecting a 1558 bp fragment of *O2.51* (positions 463-2021) for insertion as an inverted repeat in the pHANNIBAL plasmid. Independent cell lines were transformed with either the empty pHANNIBAL vector or pHANNIBAL carrying the inverted *O2.51* repeat. Out of 39 generated stable cell lines 8 cell lines (9, 12, 17, 18, 25, 27, 31, 33) had different expression patterns in comparison with controls, so we kept them for further analysis (Fig. S5). Since silencing cannot be confirmed by Northern blot, an RT-PCR was done with *O2.51* cDNA-specific primers. A primer pair was designed based on the part of the *O2.51* gene that was not included in the pHANNIBAL-*O2.51* construct (position 1- 462). Screening of those 8 selected cell lines yielded a single line with lower expression of O2.51, i.e. RNAi-*O2.51* line 27 (Fig. S6).

The silenced cell line together with 2 control cell lines were also treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 3 time points (0, 0.5, 6 h). *ORCA2* showed aberrant expression in all lines since it was not induced by MeJA. *ORCA3* showed aberrant timing of induction in one control line. The expression of *TDC* and *STR* was slightly but notably higher in the silenced line compared to both control lines (Fig. S7). While the replicate lines are missing to confirm this result, this finding is in line with the notion that the reduction of the O2.51 protein amount results in higher amounts of ORCA proteins. In the knockdown cell line there was an accumulation of *STR* and *TDC* after 6 hours which was a bit higher than controls.

O2.51 appears to have a negative effect on ORCA2 and ORCA3 activities in the transient trans-activation assay

The yeast-two hybrid experiment (Fig. 2) showed that FL-O2.51 interacted with Δ5ORCA2. Therefore, the effect of O2.51 on the activity of ORCA2 was tested in a transient transactivation assay. In addition, the possibility that O2.51 might also affect the activity of ORCA3 was investigated, because ORCA3 acts redundantly with

ORCA2 in the MIA pathway (Peebles et al., 2009; Li et al., 2013). To assess whether *O2.51* overexpression affects ORCA activity, *ORCA2* and *ORCA3* were transiently expressed in *C. roseus* cells in the absence or presence of *O2.51* co-expression. ORCA activity was measured by transactivation of the *STR* promoter, which was coupled to the *GUS* reporter gene to facilitate promoter activity measurements. Furthermore, all tested effector proteins were expressed using the CaMV *35S* promoter. Controls contained the empty vectors to equalize plasmid amounts in all co-bombardments. *ORCA2* showed a higher level of activation than *ORCA3*. O2.51 appeared to reduce the activities of ORCA2 and ORCA3. The experiment suggested that O2.51 has a negative effect on the activities of ORCA2 as well as ORCA3, although this effect of O2.51 was not significantly different compared to the control in statistical analysis using the Student *t*-test (Fig. 5). The experiment was repeated four times giving consistently similar results about the tendency of O2.51 to reduce the activities of ORCA2 and ORCA3 (Fig. S8).

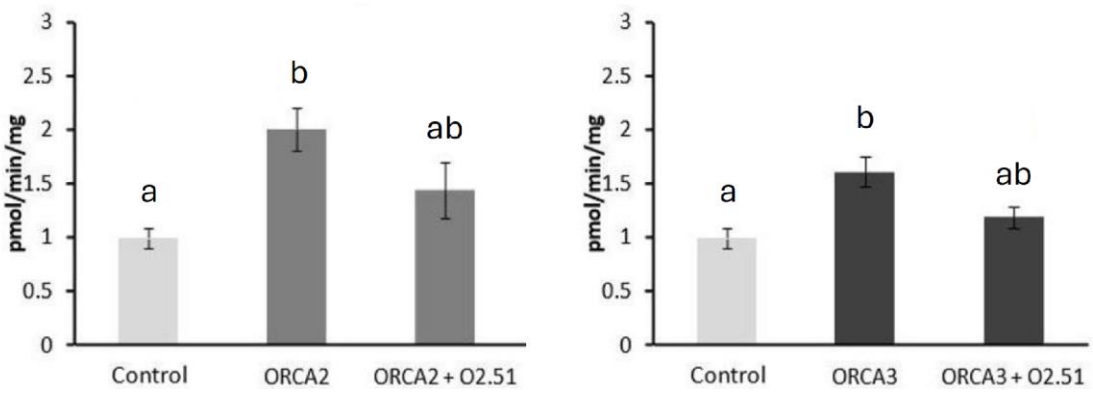

Figure 5. Effect of FL-O2.51 on ORCA2 and ORCA3 activities in transient assays. *C. roseus* MP183L cells were transiently co-transformed with 2 µg *STR-GUS* reporter construct and 4 µg of each effector plasmid. Total effector amount was adjusted to 8 µg in all transformations using pMOG184 vector plasmids (for *ORCA2* and *ORCA3*) and pRT101 vector plasmids (for FL-*O2.51*). Bars represent means +/- SEM (n=3). Letters indicate statistically significant values (P<0.05) according to one-way ANOVA with Tukey post hoc test. GUS activities are shown in pmol MU/min/mg protein.

Discussion

The alkaloids vincristine and vinblastine are derived from the JA-responsive MIA biosynthetic pathway in *C. roseus*. It has been shown that MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by a transcription factor cascade targeting the AP2/ERF-domain transcription factors ORCA2 and ORCA3, which regulate in turn a subset of alkaloid biosynthesis genes including *TDC* and *STR* (Zhang et al., 2011). These TFs are regulators of the middle part of the MIA pathway (Memelink and Gantet, 2007). Here, we found that the F-box protein O2.51 interacts with ORCA2 in yeast. Sequence analysis revealed that the F-box protein O2.51 shares 91% aa identity with the F-box proteins EBF1 and EBF2 from *A. thaliana*. This supported our hypothesis that O2.51 might facilitate the degradation of ORCA2 and ORCA3 through the 26S proteasome, much like how EBF1/2 target the transcription

factors EIN3/EIL1 for degradation (Hao et al., 2021). When ethylene is present, EBF1/2 become inactive, and EIN3/EIL1 are not subjected to degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). A comparable mechanism could be in play for the interaction between O2.51 and ORCAs.

Full-length O2.51 interacted with Δ5ORCA2 but not with Δ5ORCA3 in yeast. In trans-activation assays, O2.51 had negative effects on the activities of both ORCAs. One option is that O2.51 does not interact with the truncated ORCA3 but does interact with the full-length ORCA3, which may be testable by swapping the vectors in the yeast two-hybrid assay. Another option is that ORCAs might need a post-translational modification. For example, an attractive theory is that phosphorylation by the casein kinases I studied in Chapter 4 increases the affinity for O2.51. This is testable by combining ORCAs, O2.51 and CKIs in the transactivation assay, or by doing a yeast tri-hybrid assay. The co-expression of O2.51 with either ORCA2 or ORCA3 resulted in a decreased activation of the *STR* promoter although this was not statistically significant in a Student *t*-test due to relatively high variation between replicates. This might be solvable by including a reference gene in the assay to correct for transformation and extraction efficiencies thereby reducing variation.

MeJA induces the expression of all known MIA biosynthesis genes and both ORCA2 and ORCA3 (Menke et al., 1999; Van der Fits and Memelink, 2000; Miettinen et al., 2014). MeJA treatment did not induce the expression of the *O2.51* gene. There is still a possibility that the activity of O2.51 is regulated by JAs at the protein level, for example, O2.51 activity could be negatively regulated by JAs, similar to how ethylene has a negative effect on EBF1/2 activity.

We did not succeed in the generation of reliable overexpression lines. Many lines appeared to express truncated versions of the *O2.51* gene. In addition, gene expression between overexpression and control lines was highly variable which made it difficult to draw any firm conclusions.

We were able to generate only a single silencing line. Interestingly the expression of the ORCA target genes *TDC* and *STR* seemed to be higher in line with our working hypothesis. Maybe knocking out *O2.51* with the Crispr-Cas technique is a more promising approach.

Material and methods

Plasmid constructs

For overexpression of the F-box protein O2.51, the *O2.51* open reading frame (ORF) was PCR amplified with the primers 5'-GGA ATT CAA ATG TCT AAA GTC TTT GAT TTC GC-3' and 5'-C CTC GAG GGA TCC TTA GTA AAG GAT ATC ACA CCT CC-3' using a pACTII cDNA library as template, digested with EcoRI/XhoI and cloned initially in pJET1.2 (ThermoScientific). Using pJET1.2 clone - #2 the *O2.51* ORF was excised with EcoRI/BamHI and cloned in pRT101 (Töpfer et al., 1987) and digested with EcoRI/BamHI. The *O2.51* RNAi construct consisted of an inverted repeat of an *O2.51* fragment (463-2021) in pHANNIBAL (Wesley et al., 2001) to produce

double-stranded RNA of the inserted sequence, triggering post-transcriptional silencing. First, a BglII (pos. 463)-XbaI (pos. 2021) fragment was isolated from pRT101-O2.51 and cloned in pHANNIBAL digested with BamHI/XbaI. Next, a BglII-XhoI (463-2021) fragment was isolated from pJET1.2-O2.51#2 and cloned in pIC-19R (Marsh et al., 1984) digested with BamHI-XhoI. The last step was the isolation of the EcoRI-XhoI fragment from pIC-19R-O2.51BX and cloning in pHANNIBAL-O2.51RI. For identification of plasmids containing the inverted repeat, minipreps were checked with EcoRI/XhoI and positive minipreps were additionally digested with XhoI/XbaI. The FL-O2.51 ORF was excised with EcoRI/XhoI from pJET1.2 clone #2 and cloned in the vector pACTII with EcoRI/XhoI and is referred to as plasmid construct pACTII-FL-O2.51. All the plasmid constructs were isolated by transformation into the *Escherichia coli* strain XL1-Blue and were purified according to the QIAGEN plasmid Midikit 100® Purification Protocol.

Cell culture, stable transformation, treatments

Catharanthus roseus cell suspension line MP183L was maintained by weekly 5-fold dilution in 20 ml of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 25˚C in a 16/8 hour light/dark regime at 200 µE m^{-2} S^{-1} at 60% relative humidity on a rotary shaker at 120 rpm. For stable transformation of cell line MP183L,the plasmid construct of interest was co-transformed with the plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV *35S* promoter in a ratio of 4 to 1 by particle bombardment (Van der Fits and Memelink, 1997). Transgenic cells were selected on a solid LS medium containing 50 µg/mL hygromycin-B and individual transgenic calli were converted to cell suspensions. Cells were incubated overnight with either 10µl DMSO (D) or 10 µM Estradiol (E). Cells were then harvested by vacuum filtration, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80°C.

RNA isolation and Northern blot analysis

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose, 1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washing of blots were performed as described (Memelink et al., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *STR* (X61932), *TDC* (M25151), *RPS9* (AJ749993).

RT-PCR analysis of *O2.51* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5’-GATCTGCATTCCCAAACCCG-3’ as

forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as reverse primer. The expected size of the PCR product is 2200 bp (Fig. S3).

RT-PCR analysis of *O2.51* silencing

One µg of total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The silencing construct for *O2.51* was made by selecting a 1558 bp fragment of *O2.51* (positions 463-2021) for insertion as an inverted repeat in pHANNIBAL. For doing the RT-PCR the part that was not inserted in pHANNIBAL (1-463) was used as a template. 5'-ACCTGGGGGACAAGAGAAGA-3' was used as forward primer and 5'-ACAGGATCAGCCACAACTCC-3' as reverse primer. PCR of the *RPS9* gene was also done. *RPS9* forward primer was TCCACCATGCCAGAGTGCTCATTAGG and reverse primer TCCATCACCACCAGATGCCTTCTTCG (Fig. S6).

Isolation of plasmids and probes

Plasmid DNA for the bombardment was isolated with the QIAGEN plasmid Midikit 100® according to the manufacturer's instructions. Plasmids for analysis during cloning procedures were isolated using a laboratory protocol. The plasmid miniprep protocol was modified from Birnboim and Doly (1979). The probes *ORCA2* and *ORCA3* were amplified with PCR and afterwards isolated with a Gel Purification Kit (Thermo Scientific). PCR was done with a Thermo Scientific Phusion High-Fidelity DNA Polymerase using M13 forward and M13 reverse primers.

Yeast two-hybrid screening

Saccharomyces cerevisiae strain PJ69-4A was grown on yeast extract peptone dextrose (YPD) agar for 3 days at 30°C. This agar was made with 20 g/l Difco peptone, 10 g/l yeast exact, 20 g/l glucose adjusted to pH 5.8 with HCl, with 18 g/l agar for solid medium. Yeast colonies were inoculated in YPD medium and grown overnight in a shaker at 30°C. The yeast transformation was done according to Gietz et al. (2007). Transformants were plated on Synthetic Minimal Media (SD) lacking Leu, Trp (-LT) solid medium and grown at 30°C for 6 days. Spot assays were done by inoculating 3 colonies per plate in 1 mL SD-LT liquid media and growth overnight at 30°C. Afterwards, cultures were 10 times and 100 times diluted in SD medium lacking Leu, Trp, and Ade (SD-LTA) media. Six combinations in 2 dilutions were spotted on an SD-LTA plate and an SD-LT plate as a control. Plates were incubated for 5 days at 29ºC.

Transient expression assay

Particle bombardment was used to transform the constructs into cells of *C. roseus* cell suspension line MP183L as described (Van der Fits and Memelink, 1997). Cells were co-bombarded with 2 µg of an *STR-GUS* reporter construct carrying the *STR* promoter derivative BH (Menke et al., 1999) and 8 µg of effector plasmids. Twenty hours after

bombardment, cells covering half of the Petri dish were harvested and frozen in liquid nitrogen. Co-transformation of the reporter plasmid with the empty overexpression vectors served as controls. The GUS activity in *C. roseus* cell suspension line samples was measured by a fluorometric assay.

Acknowledgements

We thank Guillaume Chatel for construction of pAS2-1-Δ5ORCA2, and initial auto-activation tests. M.D. was partially supported by the Iranian Ministry of Science, Research, and Technology.

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Supplementary information

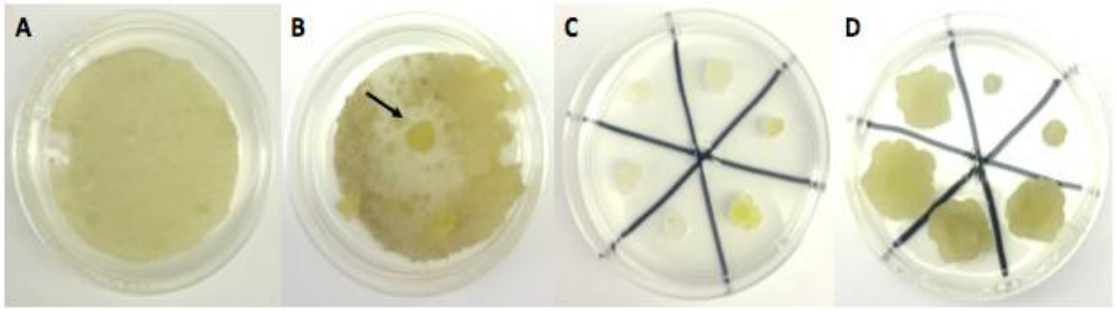

Figure S1. Generating transgenic *C. roseus* cell lines. (Diameter petri dish: 60 x 15 mm) (A) *C. roseus* cells on a filter paper one day after bombardment. (B) After 4 weeks calli had grown, arrow indicates a callus. (C) Grown calli were transferred to new media. (D) After another 4 weeks calli had grown big enough to be transferred to liquid media.

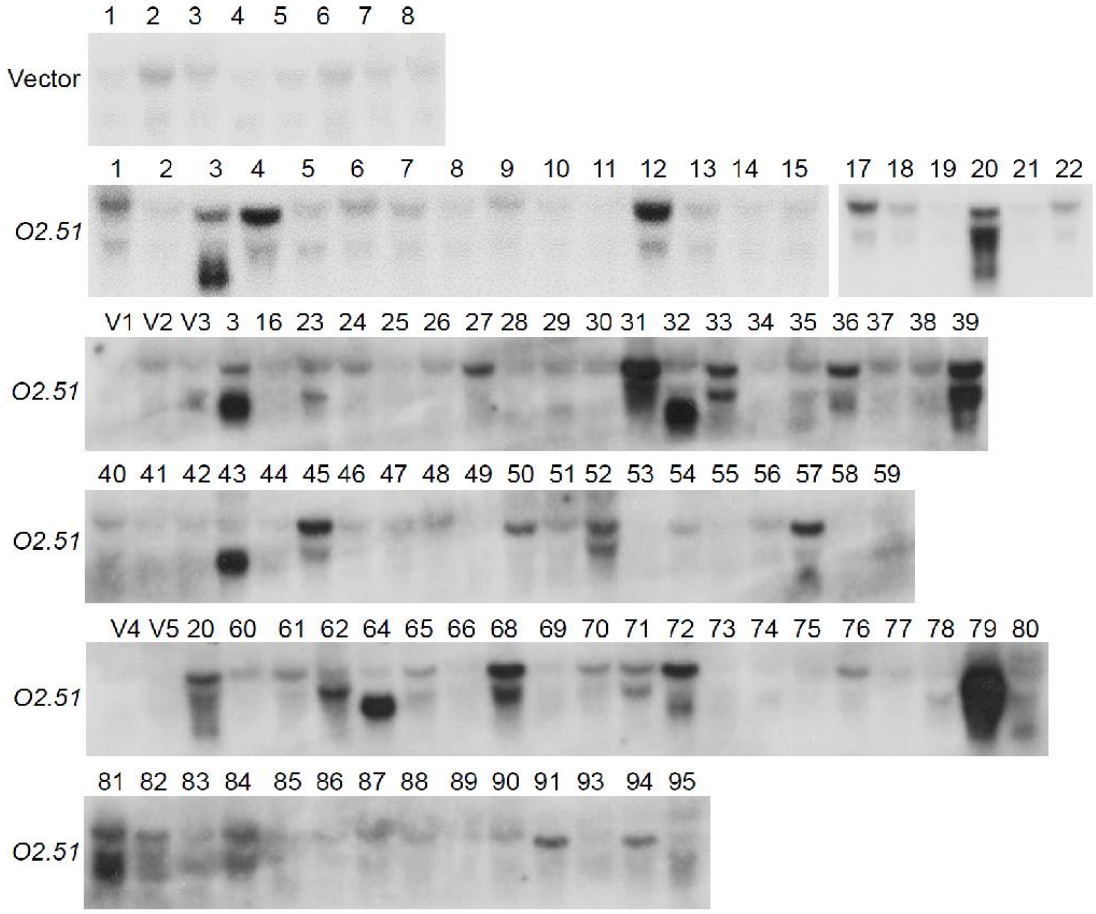

Figure S2. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* overexpression construct. Northern blots containing identical amounts of total RNA from independent control lines or lines transformed with *O2.51* cDNA overexpression construct, were hybridized with the *O2.51* cDNA probes. Vector lines were transformed with empty pRT101.

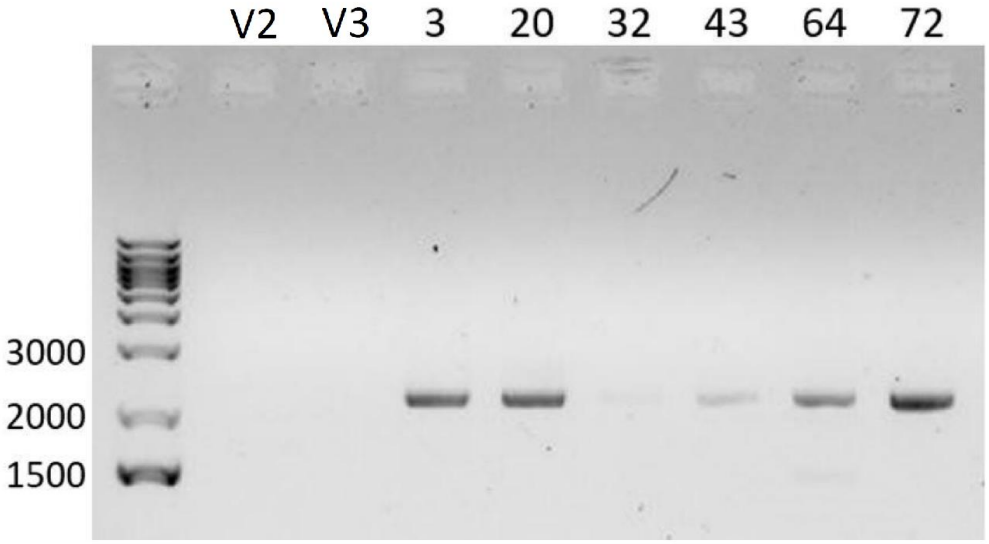

Figure S3. RT-PCR analysis of *O2.51* overexpression. Total RNA from independent cell lines were reverse transcribed (RT). The RT products were then PCR amplified. The expected size of the PCR product is 2200 bp. V indicates empty vector control lines.

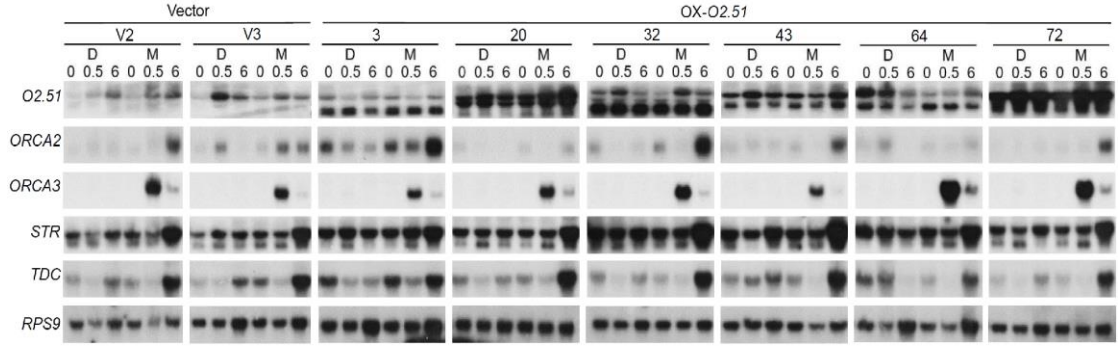

Figure S4. *O2.51* is not induced by MeJA**.** Northern blot showing *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* mRNA levels in independent transgenic control cell lines (V2, V3) and overexpression cell lines (3, 20, 32, 43, 64, 72). Cells were incubated for 0, 0.5, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* cDNAs.

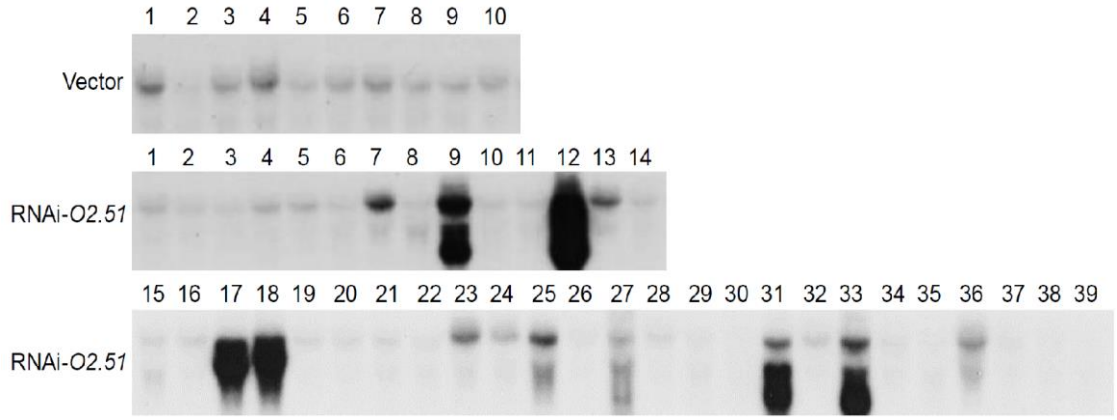

Figure S5. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an *O2.51* silenced construct. Northern blots containing identical amounts of total RNA from each line were hybridized with the *O2.51* cDNA probes. Vector lines were transformed with pHANNIBAL-empty.

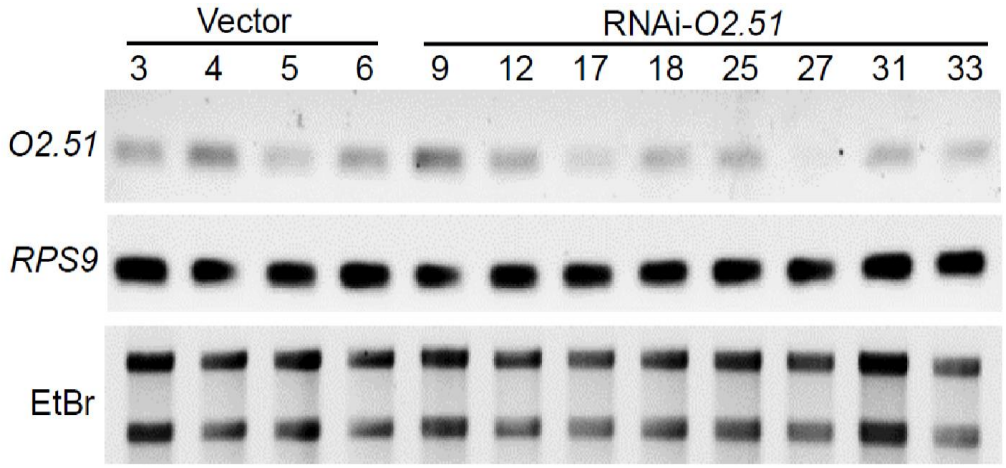

Figure S6. RT-PCR analysis of *O2.51* silencing. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. PCR of *RPS9* gene was included as a control. The Ethidium Bromide (EtBr) stained gel of input RNAs is shown as a loading control.

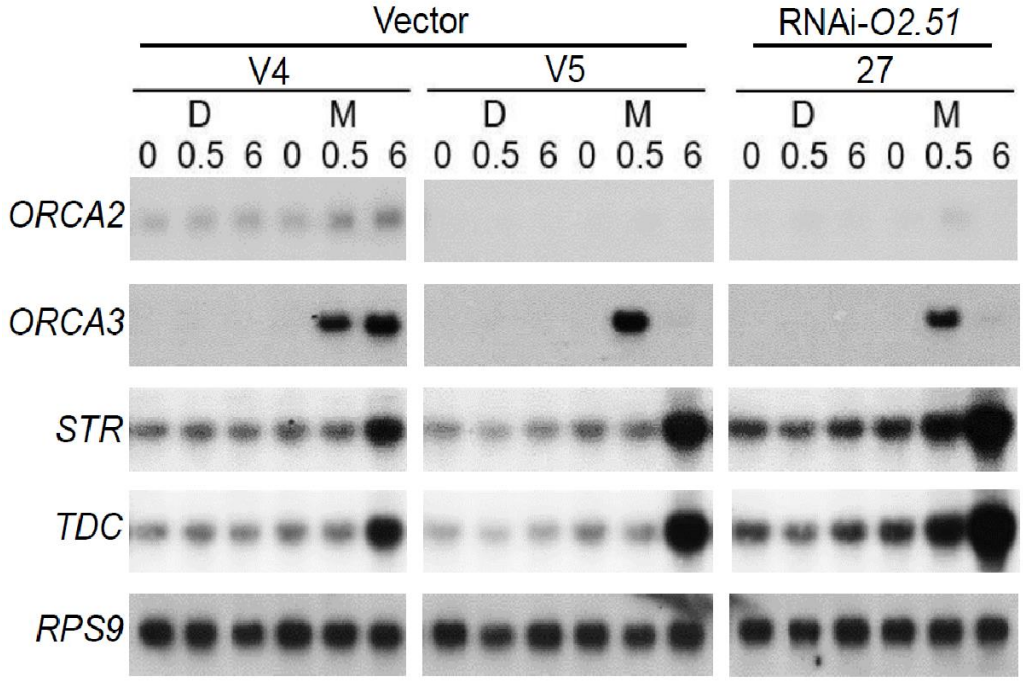

Figure S7. Time course analysis of gene expression in control cell lines V4 and V5, and in RNAi-*O2.51*. Northern blot showing *O2.51*, *ORCA2*, *ORCA3*, *STR*, *TDC* mRNA levels in independent transgenic cell lines. Cells were incubated for 0, 0.5, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *O2.51*, *ORCA2*, *ORCA3*, *STR*, and *TDC* cDNAs.

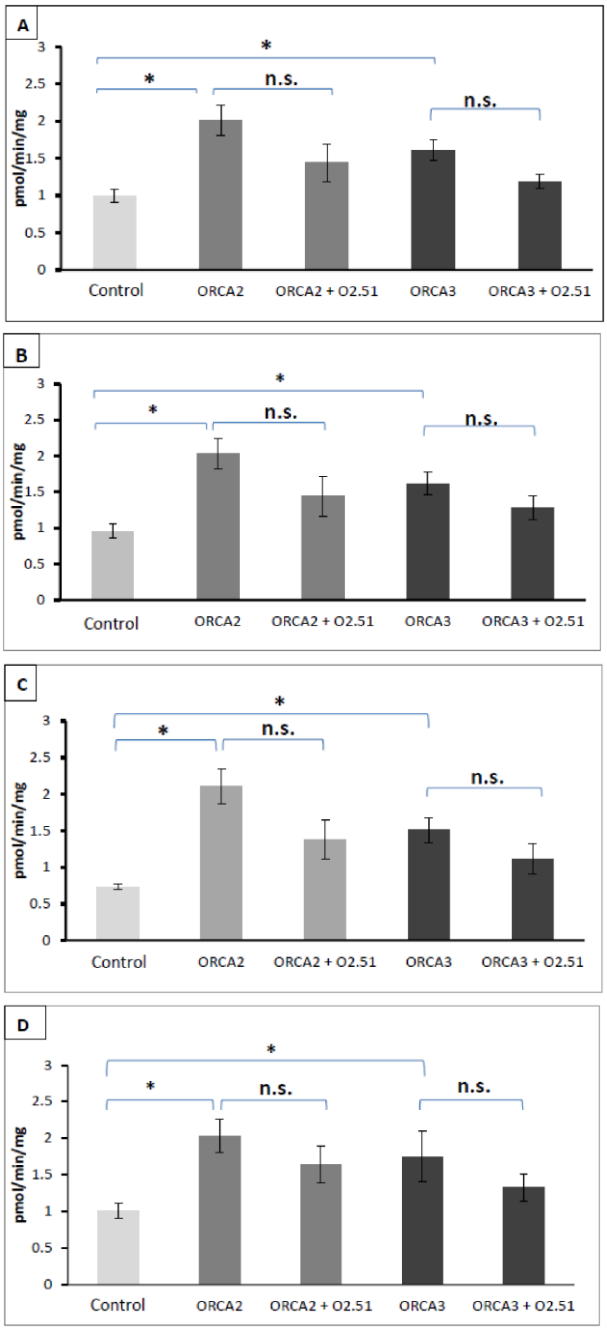

Figure S8. Effect of FL-O2.51 on ORCA2 and ORCA3 activities in transient activation assays. *C. roseus* MP183L cells were transiently co-transformed with 2 µg *STR-GUS* reporter construct and 4 µg of each effector plasmid. Total effector amount was adjusted to 8 µg in all transformations using pMOG184 vector plasmid (for ORCA2 and ORCA3) and pRT101 vector plasmid (for FL-O2.51). Bars represent means +/- SEM (n=3). Asterisks represent Student's *t*-test significance (*, $P<0.05$), while n.s. stands for not significant between pairs indicated with brackets. GUS activities are shown in pmol MU/min/mg protein. Panel A to D represent repetitions of the same experiment.

Chapter 4

Roles of *casein kinase I* in the regulation of monoterpenoid indole alkaloid biosynthesis genes in *Catharanthus roseus*

Mina Darehei, Debora Vom Endt, Laurens T.C. Boelens, Jierui Zhao and Johan Memelink

Plant Cluster, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

Abstract

The medicinal plant *Catharanthus roseus* produces more than 130 monoterpenoid indole alkaloids (MIA) including the well-known antineoplastic compounds vinblastine and vincristine. Jasmonic acid (JA) is a plant signaling molecule that stimulates the expression of MIA biosynthesis genes. The transcription factors Octadecanoid derivative-Responsive *Catharanthus* AP2-domain protein 2 and 3 (ORCA2 and ORCA3) regulate the middle part of the MIA pathway including the *Strictosidine synthase* (*STR*) and *tryptophan decarboxylase* (*TDC*) genes. A yeast two-hybrid screening resulted in the identification of ORCA3 interacting proteins that belong to the casein kinase I (CKI) family. *C. roseus* expresses a small family of four related CKIs. Here, we investigated the role of CKIs, especially CKI-1, in the regulation of ORCA transcription factors. CKIs were able to phosphorylate the ORCAs *in vitro*. CKI-1 had a strong inhibiting effect on the trans-activating activity of the ORCAs *in vivo*. GFP fusion studies showed that all four CKIs were localized in the nucleus. Based on these observations, we hypothesize that ORCA activity is modulated by CKIs.

Keywords: *Catharanthus roseus*, Casein kinase I, ORCA2, ORCA3

Introduction

Plants engage in the synthesis of a diverse array of secondary metabolites as adaptive responses to challenging environmental conditions. These metabolites serve not only as defensive agents but also contribute significantly to the overall growth and development of plants (Raina et al., 2012).

Catharanthus roseus, commonly known as Madagascar periwinkle, is recognized for the production of approximately 200 alkaloids, predominantly monoterpenoid indole alkaloids (MIAs). Notable among these are the anticancer drugs vinblastine and vincristine (Paul et al., 2016; Yang et al., 2023).

Jasmonates (JAs), including Jasmonic acid (JA) and related oxylipins, function as crucial signaling molecules in plant stress responses and diverse developmental processes. Additionally, they play a role in the regulation of ecological interactions (Wasternack and Strnad, 2019; Li et al., 2021). JAs exert their influence on the MIA pathway by activating regulatory transcription factors, where members of the APETALA2/Ethylene Response Factor (AP2/ERF)-domain transcription factor family have emerged as key regulators in JA-responsive gene expression of the middle part of the MIA pathway (Liu et al., 2015; Memelink, 2009). Octadecanoid-Responsive *Catharanthus* AP2-domain protein 2 (ORCA2) (Menke et al., 1999) and ORCA3 (Van der Fits and Memelink, 2000) belong to a small family of related AP2/ERF-domain transcription factors that also includes ORCA4, ORCA5 (Paul et al., 2017) and ORCA6 (Singh et al., 2020). They are believed to be partially functionally redundant but also have specific functions and they regulate the JA-responsive expression of the middle part of the MIA pathway genes, including *tryptophan carboxylase* (*TDC*) and *strictosidine synthase* (*STR*). The *ORCA* genes are transcriptionally regulated by JA via the key regulators CrMYC2 (Zhang et al., 2011) and the JAZ repressors (Patra et al., 2018).

Another important mechanism for controlling transcription factor activity is post-transcriptional regulation (Vom Endt et al., 2002). Protein phosphorylation, catalyzed by protein kinases, is one of the major posttranslational modifications involved in the activity, stability and localization of transcription factors.

Here we report the identification of a small family of four related Casein Kinase I (CKI) proteins in *C. roseus* which can interact with ORCA3 in yeast and can phosphorylate ORCA2 and ORCA3 *in vitro*. CKI-1, the family member studied in more detail, had a strong inhibitory effect on the activity of the ORCAs, suggesting that phosphorylation by CKIs plays a pivotal role in modulating ORCA activity.

Results

Yeast two-hybrid screening for ORCA3-interacting proteins

Since the full-length ORCA3 sequence resulted in strong auto-activation in yeast, the truncated version Δ5ORCA3 lacking an N-terminal acidic domain and without auto-activation activity was cloned in the yeast expression vector pAS2-1 creating a fusion protein between the truncated ORCA3 and the GAL4 DNA-binding domain. This construct was used to screen a cDNA library of elicitor-treated *C. roseus* suspension cells in the yeast expression vector

pACTII in yeast strain PJ69-4A. cDNA-encoded proteins are expressed as fusions with the GAL4 activation domain. PJ69-4A contains two nutritional markers (*HIS3* and *ADE2*) and a reporter gene (*MEL1)* driven by the GAL4-controlled *GAL1*, *GAL2* and *MEL1* promoters respectively (James et al., 1996). The use of different promoter-reporter genes eliminates false positives. The number of positive yeast transformants in each step of the screening is listed in Table 1. In the first round of screening, the transformed cells were plated on a medium lacking histidine. Colonies from these plates were then re-streaked on a medium lacking adenine. Growing yeast cells were then patched on plates containing X-α-Gal to detect α-galactosidase activity conferred by the GAL4-controlled *MEL1* gene. Plasmids were extracted from yeast colonies that turned blue on the last medium as a consequence of *MEL1* gene activation and transformed to *E.coli* for plasmid preparation. pACTII plasmids containing different cDNA inserts were then re-transformed to PJ69-4A together with the empty pAS2-1 plasmid, or with pAS2-1 containing Δ5ORCA3. Growth was compared and 48 pACTII clones, which were able to confer growth only in the presence of the bait, were considered true positives (Table 1). Based on Southern blot cross-hybridization experiments positive clones were grouped in classes (Table 2). The unique clone and the longest clone from each cross-hybridization class were further characterized by sequencing, and sequence comparison with the NCBI DNA database (http://www.ncbi.nlm.nih.gov) (Table 2). Two classes of Δ5ORCA3 interacting clones, O3.III and O3.IV, showed homology to casein kinase I (CKI). These two classes did not cross-hybridize, indicating that they code for different members of this family of serine/threonine protein kinases.

Table 1. Number of yeast transformants screened using the yeast two-hybrid system and the number of positive clones that were isolated in each step of the screening with Δ5ORCA3 bait.

Bait	**Δ5ORCA3**
Transformants screened	6.0×10^5
Histidine selection	313
Adenine selection	173
X-α-gal screening	156
Retransformation	48

Table 2. Classification of the positive clones found using Δ5ORCA3 as bait in yeast two-hybrid screening. Classes of cross-hybridizing clones are indicated. The column "#clones" represents the number of members of each class. Homologies to proteins in the NCBI database are indicated.

Classes	**# clones**	**Homologous to**
I	25	Centromere/Kinetochore protein ZW10
II	8	Glutathione S-transferase
III	5	Casein Kinase I
IV	9	Casein Kinase I
V	1	Homeodomain-like Protein

Sequence analysis of CKI

The *C. roseus* RNAseq database at ORCAE (bioinformatics.psb.ugent.be/orcae) was searched for sequences with homology to CKIs. It turned out that this database contained four expressed members of the CKI gene family, termed CKI-1 to 4, with about 70% amino acid identity. All nine members of class IV were derived from the same mRNA sequence encoding CKI-1. Class III consisted of the members CKI-2 and CKI-3. CKI-4 was not found in this Y2H screening. Nevertheless, this shows that interaction with ORCA3 is a reproducible and common feature of the CKI protein family. Conserved protein kinase domains are present in the deduced CKI aa sequences, which show strong alignment with a consensus casein kinase domain (KOG1164) from the NCBI conserved domain database (Marchler-Bauer et al., 2003), indicating that the four *C. roseus* CKI proteins are casein kinases.

CKI-1 interacts with the Ser-rich domain of Δ5ORCA3

Both CKI classes III and IV identified in this screening were found to interact with Δ5ORCA3, but not with Δ5ORCA2 (not shown). ORCA2 and ORCA3 share a highly similar AP2/ERF-domain and an acidic domain in the N-terminus. A difference between these proteins is found in the C-terminus, where a serine-rich domain is found in ORCA3 but not in ORCA2 (Fig. 1A). To investigate whether CKI-1 interacts with this part of Δ5ORCA3, a deletion derivative was constructed in which a 24 amino acid region containing 11 serine residues was deleted from the C-terminus (Δ5Δ3ORCA3, Fig. 1A). Interaction between CKI and ORCA derivatives was tested in yeast two-hybrid assays. The longest partial clone from class IV was tested for interaction with Δ5ORCA2, Δ5ORCA3 and Δ5Δ3ORCA3. CKI-1 was able to interact with Δ5ORCA3, but it lost its interacting ability when the serine-rich domain was absent (Δ5Δ3ORCA3, Fig. 1B).

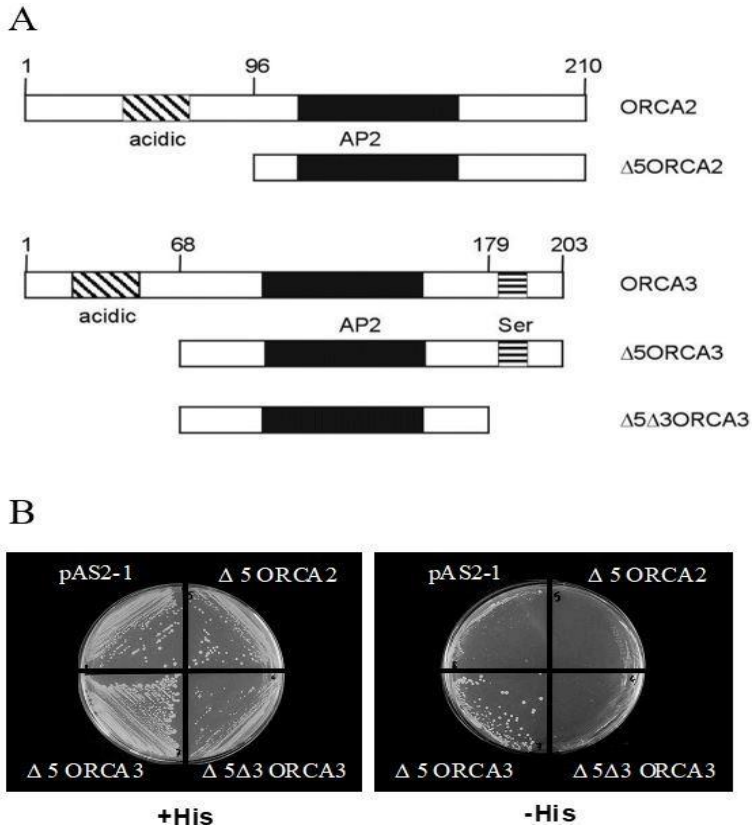

Figure 1. CKI-1 interacts with the Ser-rich domain of Δ5ORCA3. (A) Schematic representation of ORCA2 and ORCA3 and their deletion derivatives. Numbers indicate amino acid positions. AP2/ERF-domains are indicated as black boxes, and acidic domains and the serine-rich domain as hatched boxes. (B) Transformants of yeast strain PJ69-4A containing pACTII-CKI-1 (partial clone IV-113) plus empty pAS2-1 or pAS2-1 containing ORCA derivatives were grown on minimal medium with or without histidine for 5 days.

CKI phosphorylates ORCA2, ORCA3 and Δ3ORCA3

Full-length CKI-1 fused to GST was used to assay phosphorylation of His-tagged ORCA2 and ORCA3 and the Δ3ORCA3 deletion derivative. Protein mixtures of CKI-1 and ORCAs were incubated in the presence of γ-^{32}P ATP and subjected to SDS-PAGE. Gels were stained with coomassie brilliant blue and then dried and autoradiographed. The results showed that CKI-1 phosphorylated ORCA2, ORCA3 and to a lesser extent Δ3ORCA3. This indicates that the Ser-rich domain is a major phosphorylation target in ORCA3. Although ORCA2 lacks such a Ser-rich domain and did not interact with CKIs in Y2H assays, it was phosphorylated to a similar high degree as full-length ORCA3 indicating that it must have other effective phosphorylation sites. The CKI-1 preparation was strongly degraded as shown by the large amount of protein bands in lanes containing the protein. The putative full-length protein migrating at an apparent size of around 110 kDa and the two larger degradation products showed autophosphorylation activity and were probably the only active forms responsible for ORCA phosphorylation. Control incubations with GST did not lead to protein phosphorylation (Fig. 2).

The other CKIs were also tested for their ability to phosphorylate the ORCAs by incubating the full-length proteins fused to GST with full-length His-tagged ORCAs. The results showed that all four CKIs were able to phosphorylate ORCA2 (not shown) and ORCA3 (Fig. 3) to similar

levels. All CKI preparations showed extensive degradation, and only the larger fragments displayed autophosphorylation activity.

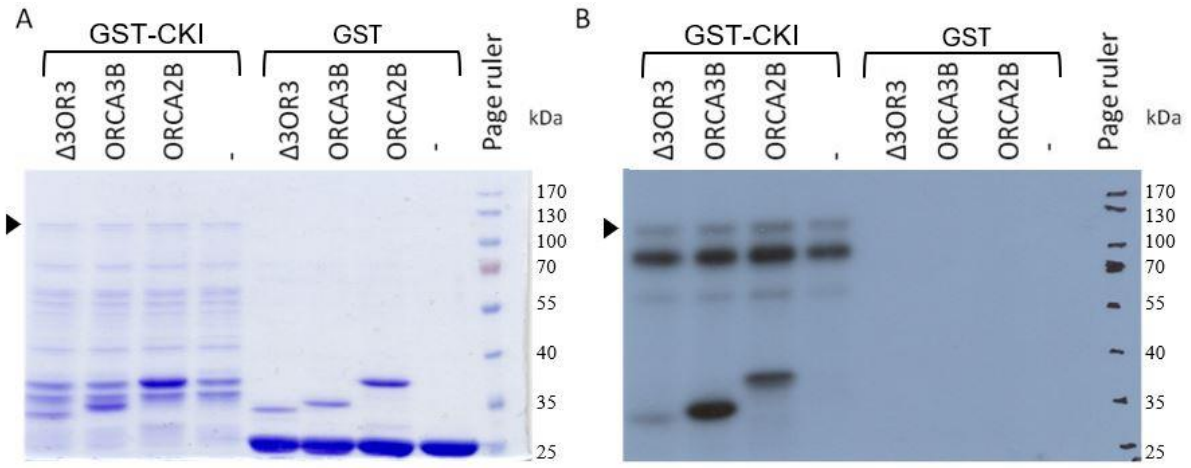

Figure 2. CrCKI phosphorylates ORCA2, ORCA3 and Δ3ORCA3. (A) Amounts of protein equal to 5 µg of ORCA and 10 µg of GST-CKI or GST as control, were incubated in the presence of γ-^{32}P ATP for 40 minutes. Samples were subjected to 10% SDS-PAGE. Staining was done with Coomassie Brilliant Blue. (B) Autoradiography of SDS-PA gel. X-ray film was exposed to the dried gel for 16 hours with a Tungstate intensifying screen at -80 °C. Protein size markers are indicated in kilo Daltons (kDa). The predicted sizes for each protein are: GST=25 kDa, ORCA2=24 kDa, ORCA3=22.3 kDa, Δ3ORCA3=14.7 kDa, GST-CKI=104.1 kDa. The putative CKI full-length protein is indicated with a black triangle.

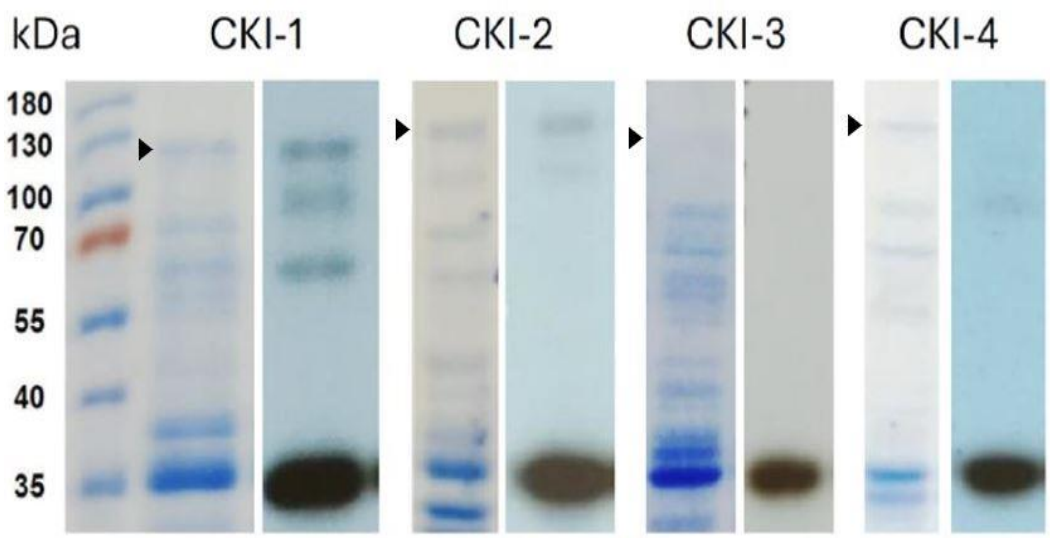

Figure 3. Phosphorylation assays of ORCA3 with GST-CKI-1/2/3/4. Reactions contained 5 µg GST-CKI-2/3/4 or 4 µg GST-CKI-1 and 3 µg ORCA3 and proceeded at room temperature for 40 min. Protein mixtures were then separated by 10% (w/v) SDS-PAGE and gels were CBB stained (left lanes in each CKI panel). Afterwards the PAA gels were dried on Whatman 3MM paper and autoradiographed using X-ray films (right lanes). Protein size markers are indicated in kilo Daltons (kDa). The predicted sizes for each kinase are 105.1 kDa (GST-CKI-1), 109 kDa (GST-CKI-2), 104.6 kDa (GST-CKI-3) and 105.3 kDa (GST-CKI-4). Putative CKI 1-4 full-length proteins are indicated with a black triangle.

Generation and analysis of stable *CKI* overexpression lines

We wanted to generate stably transformed *C. roseus* cell suspension lines overexpressing *CKI-1* and *CKI-3* to study the role of CKIs in MIA biosynthesis. The plasmid pRT101 carrying the CaMV *35S* promoter and either *CKI-1* or *CKI-3* were introduced in cell line MP183L by particle bombardment together with a plasmid carrying a hygromycin resistance gene. To generate control lines, cells were bombarded with the empty pRT101 vector. Hygromycin-resistant cell lines were analysed for *CKI-1* and *CKI-3* gene expression by Northern blot hybridization of extracted RNA to select lines that effectively overexpress *CKI-1* (Fig. S1) or *CKI-3* (Fig. S4). For *CKI-1* lines, successful overexpression lines should express a transcript with a size of 2800 nt. Some lines overexpressed a large RNA that might correspond to the expected size, while several lines expressed also smaller, possibly truncated RNAs (Fig. S1). Seven *CKI-1* cell lines were selected for further analysis. For further checking of overexpression of a full-length transcript, an RT-PCR analysis was done with a forward primer based on the first 20 bp of *CKI-1* and a reverse primer based on the CaMV terminator on the pRT101 plasmid (Fig. S2). Only CKI-1 cell lines 18 and 39 had the expected PCR product size. These cell lines together with two control cell lines were treated either with 0.1% (v/v) DMSO or 10 µM MeJA with 4 time points (0, 0.5, 3, 6 h) and analysed for expression of the *ORCA2* and *ORCA3* genes and the ORCA target genes *TDC* and *STR* (Fig. S3). Based on the hypothesis that ORCA protein phosphorylation by CKIs modulates their activities, the expectation is that *CKI* overexpression does not affect *ORCA* RNA levels, but affects the RNA levels of ORCA target genes. The analysis showed that the *ORCA* genes and their target genes were induced by MeJA as shown numerous times before. The timing of *ORCA2* gene expression was similar to the *TDC* and *STR* genes, whereas the *ORCA3* gene was expressed much earlier. The results from the Northern blot hybridization experiment showed some seemingly random variability, but we concluded that there were no consistent differences between control lines (V) and the selected overexpression lines. The *CKI-1* RNA levels also showed variable levels without a clear trend, but we concluded that the selected lines did not actually overexpress *CKI-1*. In addition, we concluded that the endogenous *CKI-1* gene was not consistently or reproducibly induced by MeJA.

For *CKI-3*, successful overexpression lines should express a transcript of 2300 nt .Several lines overexpressed a large RNA that might correspond to the expected size, while several lines expressed also smaller, possibly truncated RNAs (Fig. S4). Six *CKI-3* cell lines were selected for further analysis. RT-PCR analysis showed that all these *CKI-3* cell lines except line 15 had the expected PCR product size (Fig. S5). Analysis of *ORCA* and target gene expression did not show differences between control cell lines and the selected overexpression lines (Fig. S6). The latter lines overexpressed smaller RNAs as already observed in the initial screening but did not overexpress a larger *CKI-3* RNA of the expected size. Looking at the control cell lines we concluded that *CKI-3* expression is not reproducibly and consistently induced by MeJA.

CKI inhibits ORCA2 and ORCA3 trans-activation activity

To test the effect of CKI-1 on the activity of the ORCAs, co-bombardment experiments were performed on *C. roseus* cells with effector plasmids carrying *ORCA2, ORCA3,* Δ*3ORCA3* (Fig.

4A) and *CKI* fused to the CaMV *35S* promoter and a reporter construct consisting of the *STR* promoter derivative NH fused to the *GUS* reporter gene.

First the effective concentrations of effector plasmids and the effective ratio of CKI:ORCA3 were determined (Fig. 4B). The results show that 1 µg of pRT101-ORCA3 provided a better trans-activation than 3 µg. Co-bombardment with pRT101-CKI-1 resulted in strong inhibition of reporter gene expression even below the basal level, with similar effectiveness of the three different pRT101-CKI-1 amounts. Based on these results, 1 µg amounts of all effectors were used in Figures 4C and 4D. As reported before (Van der Fits and Memelink, 2001), the Δ3ORCA3 deletion derivative had a higher trans-activation activity than full-length ORCA3 (Fig. 4C), indicating that the Ser-rich domain has an inhibitory effect. Co-bombardment with CKI-1 still reduced reporter gene activity, but not below the basal level, indicating that CKI-1 had a lower inhibiting effect on the ORCA3 deletion derivative. Co-bombardment with CKI-1 also inhibited the trans-activation activity of ORCA2, resulting in a reporter gene expression level slightly below the basal level (Fig. 4D).

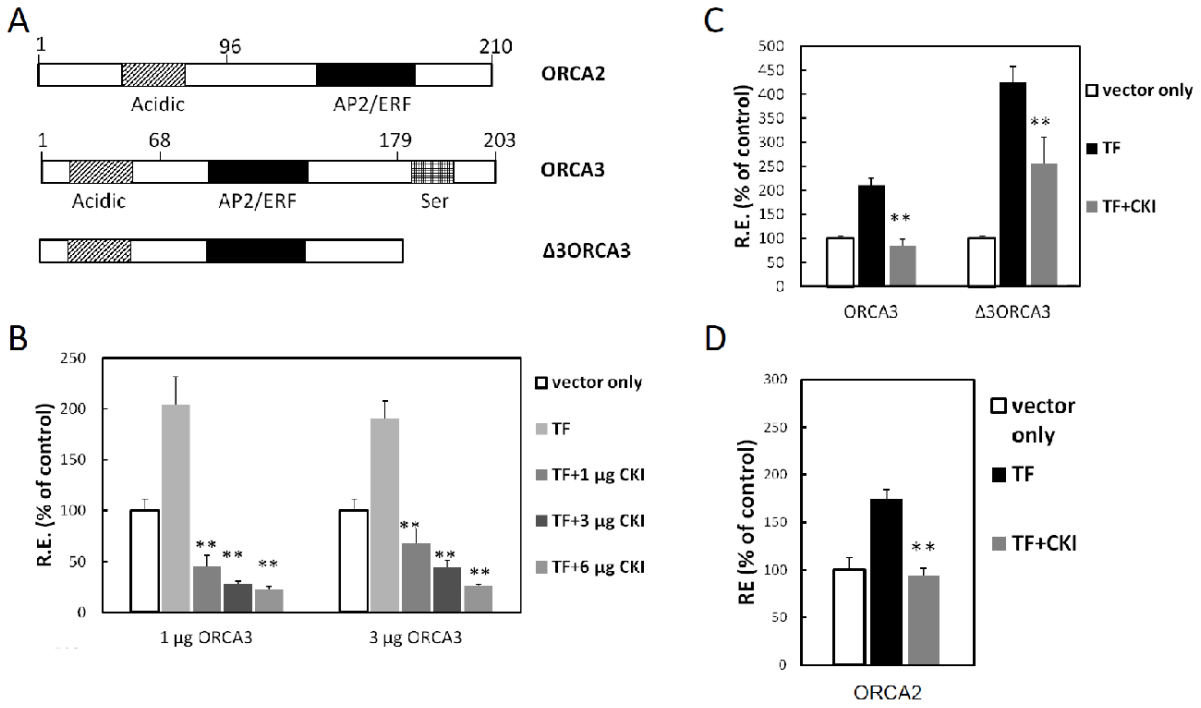

Figure 4. CKI inhibits ORCA2 and ORCA3 trans-activation activities. (A) Schematic representation of ORCA2, ORCA3 and the Δ3ORCA3 derivative. Numbers indicate amino acid positions. The AP2/ERF domain is indicated as a black box, and the acidic domain and the serine-rich domain as hatched boxes. (B) Determination of the effective ORCA3:CKI ratio. *C. roseus* cells were transiently co-transformed with *35S-ORCA*, *35S-CKI-1* and the *STR* NH promoter derivative fused to the *GUS*. Vector only indicates substitution of *ORCA* and *CKI* with the empty *35S*-plasmid pRT101. (C) Effect of CKI on ORCA3 and Δ3ORCA3 activities. *C. roseus* cells were transiently co-transformed with 1 µg pRT101-*ORCA3* or pRT101-Δ*3ORCA3* and 1 µg of pRT101-*CKI-1*. (D) Effect of CKI on ORCA2 activity. *C. roseus* cells were transiently co-transformed with 1 µg pMOG184-*ORCA2* and 1 µg of pRT101-*CKI-1*. Vector only indicates substitution of ORCA2 and CKI with the empty *35S*-plasmid pMOG184. R.E. stands for relative expression with all values normalized to % of control. Bars represent means ± standard error (n=3). ** denotes significance ($P<0.05$ with pairwise comparison using paired *t*-tests).

CKIs are localized in the nucleus of *C. roseus* cells

If CKIs are really modulators of ORCA activity, one would expect them to be localized in the nucleus. To determine the subcellular location of CKI-1/2/3/4 by fluorescence localization, we constructed the plasmids pTH2-CKI-1/2/3/4 and pTH2BN-CKI-1/2/3/4 to express the C- and N-terminal fusions with green fluorescent protein (GFP) CKI-GFP and GFP-CKI, respectively. Through polyethylene glycol (PEG) transformation, we successfully expressed the fusion proteins in *C. roseus* protoplasts. All the fusion proteins were detected in the nucleus while GFP alone was in both the cytoplasm and the nucleus (data not shown). However, compared to protoplasts from *A. thaliana*, the transformation efficiency of *C. roseus* protoplasts was much lower at about 10^{-3} to 10^{-4}, indicating that the method needs further optimization. To further check whether the GFP fusions of CK-I1/2/3/4 are indeed located in the nucleus, we co-transformed the GFP fusion plasmids with a marker plasmid which directs the expression of a red fluorescent protein (DsRED) fusion protein in the nucleus (Jasinski et al., 2002). Due to the low efficiency of transformation and for unknown other reasons, we only obtained *C. roseus* protoplasts co-transformed with pTH2-CKI1/2/3/4 and the marker plasmid. The results of confocal microscopy showed that the GFP fluorescence from CKI-GFP fusions was exactly overlapping the DsRED fluorescence from NtKIS1a-DsRED (Fig. 5), which confirmed that the location of CKI-GFP fusions was in the nucleus. Together, the fluorescence results showed that GFP fusions of CKI-1/2/3/4 were situated in the nucleus of *C. roseus* cells.

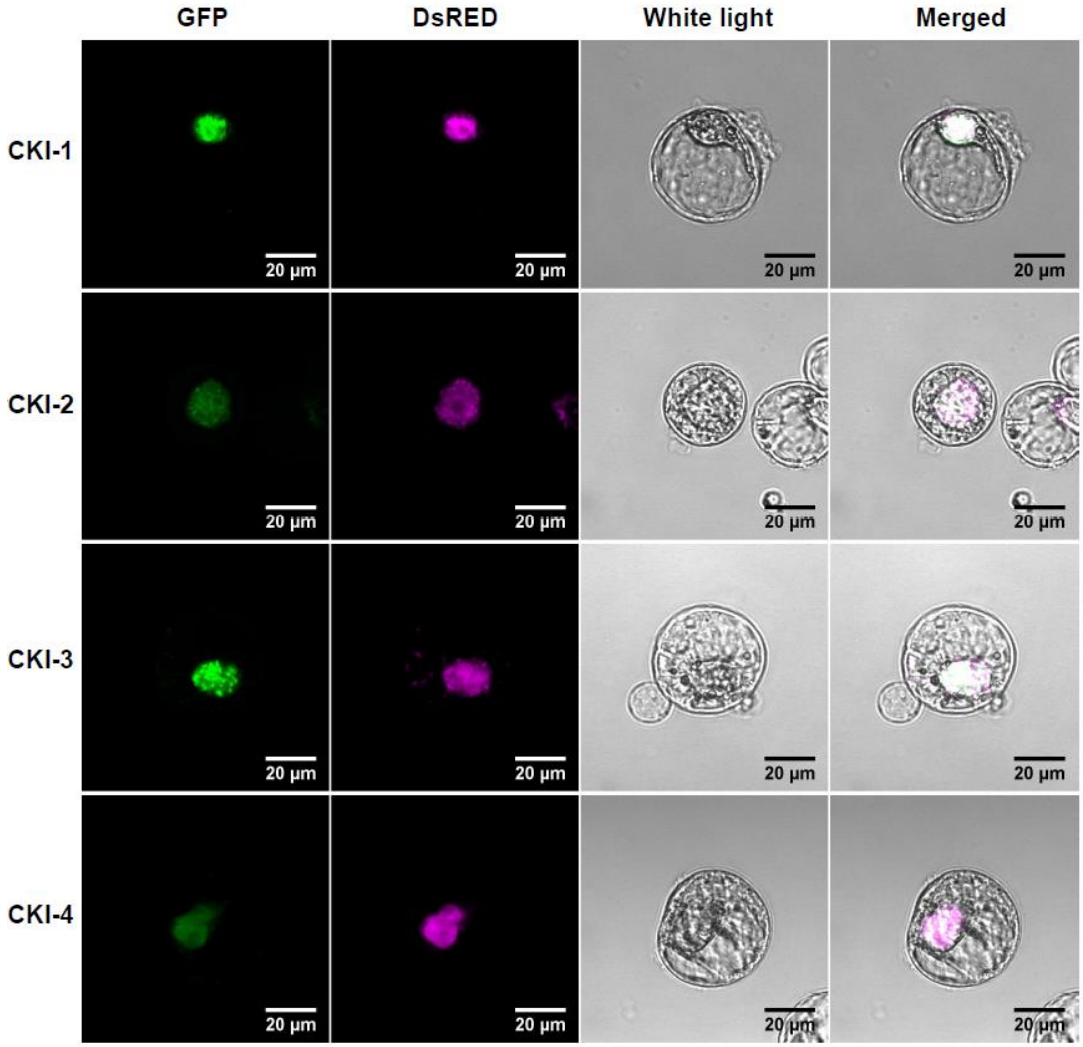

Figure 5. Fluorescence localization of CKI1/2/3/4 in protoplasts of *C. roseus*. Plasmids carrying CKI-GFP fusions were co-transformed with the NtKIS1a-DsRED marker plasmid.

Discussion

Using yeast two-hybrid screening we found a small family of related casein kinase I proteins that can interact with ORCA3. Although they do not interact with ORCA2 in the yeast two-hybrid system, they are able to phosphorylate both ORCA2 and ORCA3 *in vitro*. A Ser-rich domain in ORCA3 is necessary for interaction with CKI-1, and its removal significantly reduces the ability of ORCA3 to become phosphorylated by CKI-1. Trans-activation assays show that CKI-1 strongly inhibits the activity of both ORCA2 and ORCA3 *in vivo*. Presumably, this is due to phosphorylation of the proteins, although this remains to be demonstrated for example by using ORCA mutants that cannot be phosphorylated. However, the observation that deletion of the Ser-rich domain in ORCA3 resulted in increased trans-activation of the *STR* promoter in co-bombardment experiments (Fig. 4C; Van der Fits and Memelink, 2001) combined with the observation that this deletion derivative is less phosphorylated strongly suggest that CKI modulates the repressor activity of the Ser-rich domain in ORCA3 via phosphorylation. However, the removal of the Ser-rich domain resulted in a modest reduction in the phosphorylation level and did not abolish the inhibiting activity of CKI-1. This indicates that

ORCA3 contains other target sites for CKI. ORCA2 lacks a Ser-rich region but nonetheless is strongly inhibited *in vivo* and strongly phosphorylated *in vitro* by CKI-1 indicating that it has other efficient target sites for CKI phosphorylation.
CrCKIs were found to be localized in the nucleus, consistent with a role in transcription factor phosphorylation. The CrCKIs are about 700 aa in size and contain a large (around 280 aa) central kinase domain with the ATP binding site in the N-terminal part of the domain. Just the C-terminal of the kinase domain a monopartite nuclear localization signal (NLS) is located.
CK1 is found in all eukaryotes ranging from yeast to humans and plants, with plants having, as usual, more members. They function as monomeric proteins. In mammals, seven CK1 isoforms were identified (alpha, beta, gamma1-3, delta and epsilon), which are implicated in multiple cellular processes such as circadian rhythm, DNA repair, chromosome segregation, morphogenesis, cell cycle, intracellular trafficking and Wnt signaling.
Due to an ancient lineage duplication event, plants have evolved two subclasses of CKI1, the casein kinase 1-like (CKL) class with higher similarity to CKIs in other organisms, and a unique plant-specific group containing the Mut9p-LIKE KINASES (MLKs) (Kang and Wang, 2020). The CrCKIs belong to this plant-specific MLK subclass. The *A. thaliana* genome encodes 4 MLK members (Kang and Wang, 2020), an identical number to the four expressed CrCKIs found in *C. roseus*. CrCKI has 83% aa identity to for example AtMLK1. AtMLK1 was reported to interact in yeast with the DELLA protein REPRESSOR of *ga1-3* (RGA) and with CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a MYB-domain TF which is an integral part of the circadian clock (Zheng et al, 2018). *mlk1* mutant plants have short hypocotyls and are hyposensitive to gibberellic acid. Strangely enough, the study does not contain any phosphorylation assays. However, the rice MLK EARLY FLOWERING1 (EL1), was shown to phosphorylate the DELLA protein SLENDER RICE 1 (SLR1) to negatively regulate gibberellin signaling (Dai et al., 2010).
One question is why phosphorylation of ORCAs leads to a reduction in their activity. Phosphorylation could result in exclusion from the nucleus. Phosphorylation could reduce the affinity of ORCAs to bind their DNA target site. This was reported for the basic leucine zipper (bZIP) TF TGA5 in rice upon phosphorylation by CKII, resulting in compromised expression of defense-related genes (Niu et al., 2022). Phosphorylation could also result in degradation. An attractive theory is that phosphorylation of the ORCAs increases their affinity for the F-box protein O2.51 described in Chapter 3 of this thesis, leading to degradation via the ubiquitin-proteasome pathway.

Material and methods

Plasmid constructions

The complete sequences of four *CKI* genes (*CKI1/2/3/4*) were cloned into pRT101 (Töpfer et al., 1987). *CKI1/2/3/4* were PCR amplified using pRT101-CrCKI1/2/3/4 as templates with appropriate primers (Table S1) and then ligated to pJET1.2/blunt cloning vector (Thermo Scientific). *CKI1/2/3/4* fragments were transferred using restriction enzymes (Table S1) to pTH-2 (Chiu et al., 1996; Niwa et al., 1999) and pTH-2BN (a derivative of pTH-2 without the GFP stop codon) vectors. SalI partial digestion was used for excising the fragment of *CKI4*

from pJET1.2. All constructed pJET1.2, pTH-2 and pTH-2BN plasmids were verified by sequencing.
The coding sequences of *ORCA2* and *ORCA3* were cloned in pET-16b (Novagen) for His-tagged protein isolation according to the Novagen protocol.
For isolating GST-tagged CKI1/2/3/4 proteins, full-length sequences of *CKI1/2/3/4* were cloned into pGEX-J2 vector, a derivative of pGEX-KG (Guan and Dixon, 1991) with modified multiple cloning sites.

Cell culture, stable transformation, treatments

Catharanthus roseus cell suspension line MP183L was maintained by weekly 5-fold dilution in 20 ml of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 25°C in a 16/8 hour light/dark regime at 200 µE m^{-2} S^{-1} at 60% relative humidity on a rotary shaker at 120 rpm. For stable transformation of cell line MP183L, plasmid constructs of interest were co-transformed with the plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV *35S* promoter in a ratio of 4 to 1 by particle bombardment (Van der Fits and Memelink, 1997). Transgenic cells were selected on a solid LS medium containing 50 µg/mL hygromycin-B and individual transgenic calli were converted to cell suspensions. For RNA isolation, cells were harvested by vacuum filtration, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80°C.

RNA isolation and Northern blot analysis

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose, 1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washing of blots were performed as described (Memelink et al., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *STR* (X61932), *TDC* (M25151).
Deletion derivatives of ORCA2 and ORCA3 lacking the acidic domain in the pAS2-1 vector were used as baits for the screening. The pACTll library was prepared from *C. roseus* suspension cells treated with yeast extract for one and four hours as described by Menke et al. (1999). Co-transformation of bait and cDNA library at a ratio 1:1 was performed into yeast strain PJ69-4A according to a yeast transformation protocol modified from Gietz et al. (1992). Transformed cells were plated on a medium containing 5 mM 3AT and lacking histidine. Colonies were re-streaked on medium lacking adenine, and patched on medium containing X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; BD Biosciences – Clontech)

Fluorescence localization

Protoplasts of *C. roseus* cell line MP183L were made using a modified version of a previously described protocol for *A. thaliana* (Schirawski and Planchais, 2000). Linsmaier and Skoog medium (LS medium) was used instead of the B5 medium. For PEG transformation, the

plasmids of pTH2-CKI1/2/3/4 and pTH2BN-CKI1/2/3/4 were co-transformed with a nucleus marker plasmid NtKIS1a-DsRED (Jasinski et al., 2002), using the protocol as previously described (Sheen, 2002). The transformed protoplasts were incubated for 22 hours at 25℃ in the dark.

After incubation, the fluorescence of transformed protoplasts was examined with a confocal microscope Zeiss LSM5 Exciter/Axio Observer. For GFP visualization, the excitation wavelength was 488 nm and the emission wavelength ranged from 505 nm to 530 nm. An excitation wavelength of 543 nm and an emission wavelength ranging from 560 nm to 700 nm was used for viewing the fluorescence of DsRED.

Protein isolation

For inducing the expressions of proteins, a single colony of transgenic *E. coli* was shaken in a liquid LC medium with antibiotics at 37℃ until OD600 reached 0.5. Then 0.15 g solid isopropyl-β-d-thiogalactoside (IPTG) was added and the bacteria were grown for 3 h at 29℃ until OD600 reached around 0.8. The bacteria were collected by centrifugation and resuspended in 20 ml His binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0) for His-tagged proteins or 20 ml PBS binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) for GST-fusion proteins. Resuspended cell samples were transferred to 50 ml SS34 polypropylene tubes and then stored at -80℃. When isolating proteins by column chromatography, the samples were thawed at 37℃ and sonicated on ice until the viscosity was low. The samples were subsequently centrifuged for 20 min at 18,000 rpm in a SS34 rotor at 4℃. The supernatant was filtered with a 0.45 μm membrane (Nalgene, Catalog #190-2545) and ready for loading into the column.

His-tagged ORCA2 and ORCA3 proteins were purified using plastic poly-prep columns (Bio-Rad Catalog #731-1550) containing Ni-NTA Agarose (Qiagen). After loading the samples, the columns were washed with His binding buffer and His wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). His-tagged proteins were eluted by His elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). For isolating GST and the GST-fusion proteins of CKI1/2/3/4, Gluutathione-Sepharose 4B (Amersham Biosciences) was used for preparing the columns and purifying the proteins. GST and GST-fusion proteins loaded into columns were washed with PBS binding buffer and then eluted with glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). The isolated proteins were separated by 10% (w/v) SDS-PAGE and checked by CBB staining for quality analysis.

Phosphorylation assay

ORCA2 and ORCA3 (resuspended in His elution buffer) were incubated with GST or GST-CKI proteins (resuspended in glutathione elution buffer) in phosphorylation buffer (1 μCi [γ-^{32}P]-ATP, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) at 25℃ for 40 min. After incubation, samples were run on 10% (w/v) SDS-PAGE until the front line containing the 15-kDa marker bands of the Pageruler (Thermo Scientific) just ran out of the gel. The gel was then stained with CBB, destained and photographed and then completely dried on Whatman 3MM filter paper at 80℃ using a Model 583 Gel Dryer (Bio-Rad). The dried gels were exposed to the Fuji medical

X-ray film (Fujifilm) in a cassette with Lighting Plus intensifying screens (DuPont Cronex) at -80°C for the appropriate time depending on the radioactivity level.

RT-PCR analysis of *CKI1* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5'-ATGCCAGAGCTTCGTAGTGG-3' as the forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as the reverse primer. The expected size of the PCR product is 2800 bp (Fig. S2).

RT-PCR analysis of *CKI3* overexpression

One µg of total RNA from independent cell lines was reverse transcribed (RT). The RT products were then PCR amplified with 5'-ATGCCGGAGTTACGAAAGGG-3' as the forward primer and 5'-CAACACATGAGCGAAACCCTATAAGAACCC-3' as the reverse primer. The expected size of the PCR product is 2300 bp (Fig. S5).

Accession numbers

The sequences of *CrCKI1/2/3* can be found in the ORCAE database (Van Moerkercke et al., 2013) as Caros005148.3 (*CrCKI1*), Caros007013.3 (*CrCKI2*) and Caros009329.1 (*CrCKI3*). A partial sequence of *CrCKI4* corresponds to Caros007013.1 in the ORCAE database with the 5'part completed by the sequence of MAGPIE:cro_CRO1L1VD_velvet--Contig1393 from the Phytometasyn transcriptome database (Xiao et al., 2013). The sequences of *ORCA2* and *ORCA3* can be found in GenBank as AJ238740 and AJ251250 respectively.

Acknowledgements

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Supplementary information

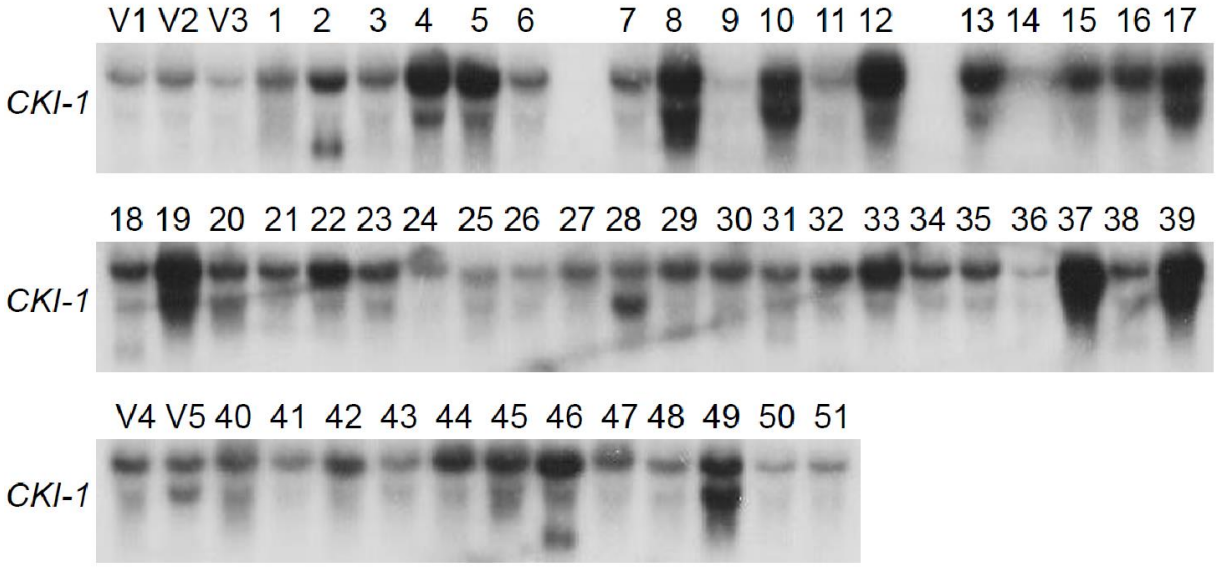

Figure S1. Expression analysis of *C. roseus* cell lines generated following particle bombardment with a pRT101-*CKI-1* construct, where *CKI-1* expression is driven by the *35S* promoter. A Northern blot containing identical amounts of total RNA from each line were hybridized with the *CKI-1* cDNA probe. All lanes were on the same blot.

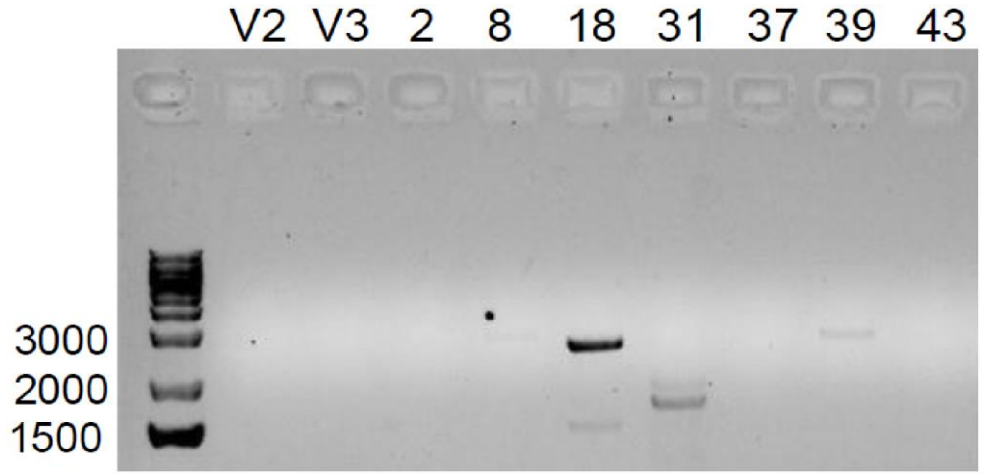

Figure S2. RT-PCR analysis of *CKI-1* overexpression. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The expected size of the PCR product is 2800 bp. V are vector control lines.

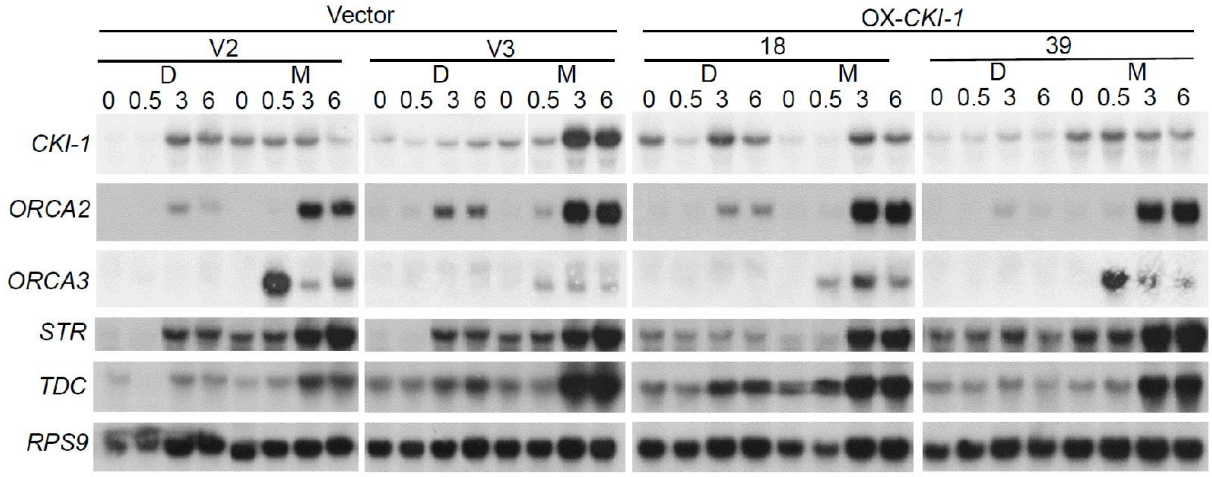

Figure S3. Time course analysis of gene expression in control cell lines V2 and V3, and OX-*CKI-1* lines. Northern blot showing *CKI-1*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* mRNA levels in independent transgenic control cell lines (V2, V3) and *CKI-1* overexpression cell lines (18, 39). Cells were incubated for 0, 0.5, 3, 6 h with either DMSO (D) or 10 µM MeJA (M). Replicate Northern blots were probed with *CKI-1*, *ORCA2*, *ORCA3*, *STR*, *TDC*, and *RPS9* cDNAs. All groups of four horizontally arranged panels were on the same blot. The white line in panel V3-*CKI-1* between M0 and M0.5 is due to removal of a leaky lane. All lanes on the group of four horizontally arranged panels were on the same blot.

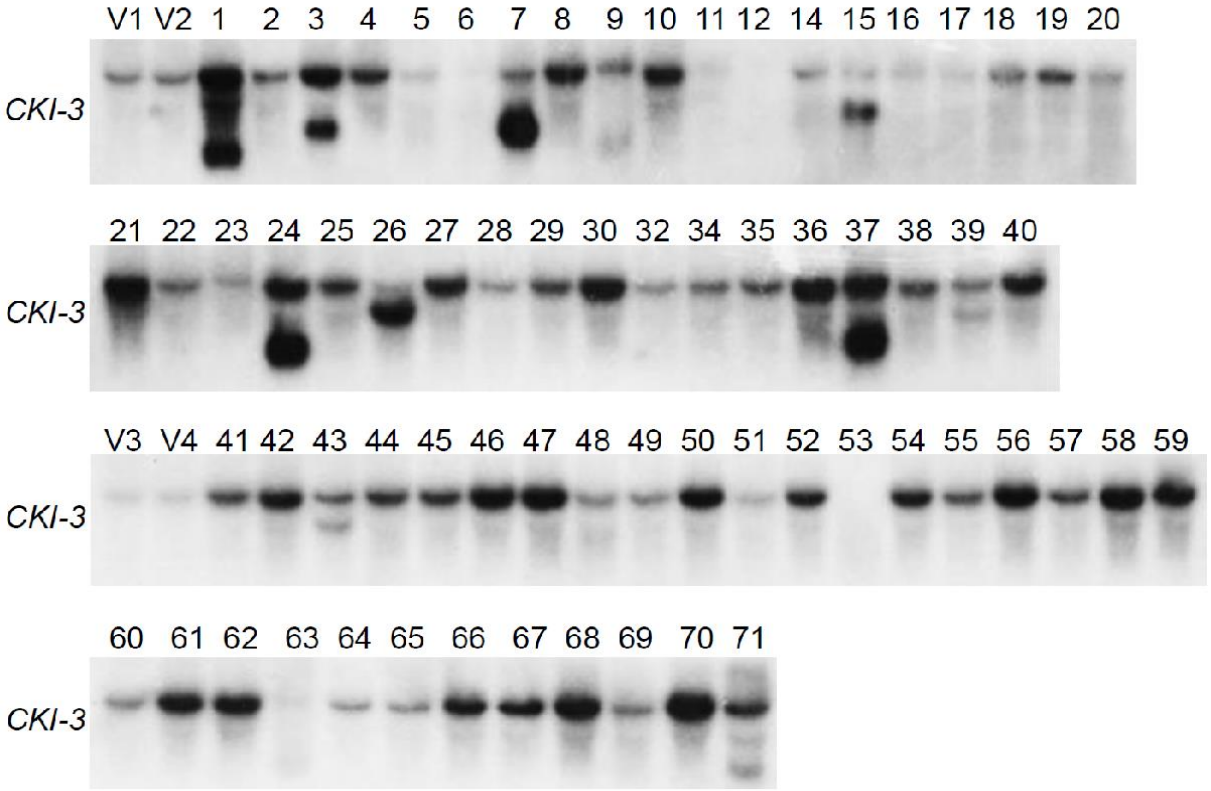

Figure S4. Expression analysis of *C. roseus* cell lines generated following particle bombardment with an pRT101-*CKI-3* construct, where *CKI-3* expression is controlled by the *35S* promoter. Northern blots containing identical amounts of total RNA from each line were hybridized with the *CKI-3* cDNA probe.

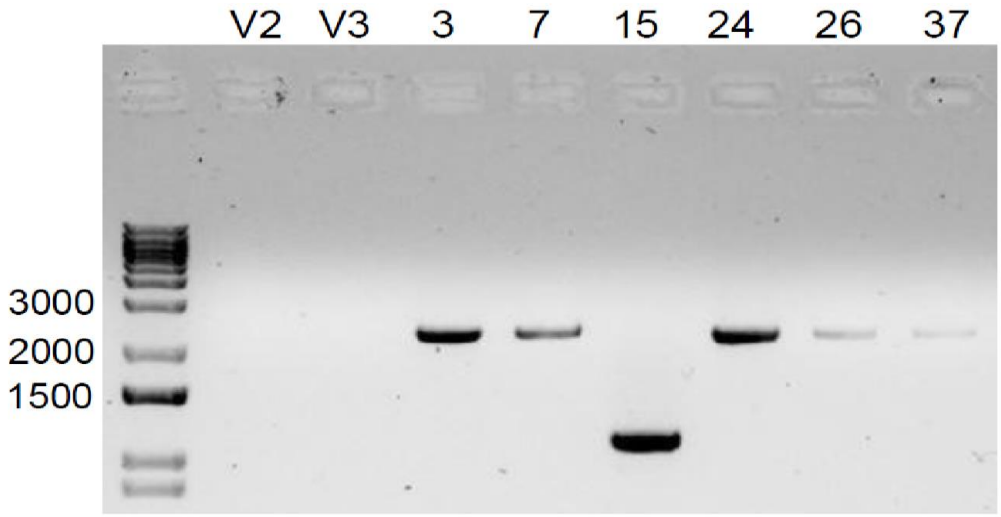

Figure S5. RT-PCR analysis of *CKI-3* overexpression. Total RNA from independent cell lines were reverse transcribed. The RT products were then PCR amplified. The expected size of the full-length PCR product is 2300 bp. V indicates vector control lines.

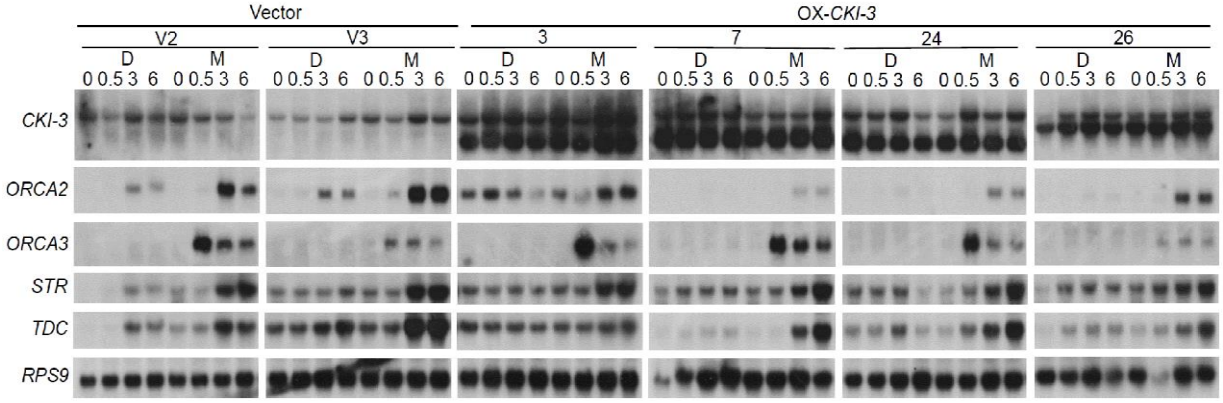

Figure S6. Time course analysis of gene expression in control cell lines V2 and V3, and OX-*CKI-3* lines. Northern blots showing *CKI-3*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* mRNA levels in independent transgenic control cell lines (V2, V3) and *CKI-3* overexpression cell lines (3, 7, 24, 26). Cells were incubated for 0, 0.5, 3, 6 h with either DMSO (D) or 10 µM MeJA (M). The Northern blot was sequentially probed with *CKI-3*, *ORCA2*, *ORCA3*, *STR*, *TDC*, *RPS9* cDNAs. All horizontally arranged panels were on the same blot.

Table S1. Templates, primers, vectors, and restriction enzymes used for constructing the plasmids for fluorescence localization.

names	templates	PCR primers and restriction enzymes	Vectors
pTH2-CKI1	pRT101-*CKI1*	5'-GCCTCGAGAAAATGCCAGAGCTTCGTAGTGG-3' 5'-ATAGTCGACGCACACCGTGCGTCCGTAACAG-3' *Xho*I/*Sal*I	pTH-2 *Sal*I
pTH2-CKI2	pRT101-*CKI2*	5'-GCGTCGACAAAATGCCTCAACTGCGTAGCGGAG-3' 5'-GCCCATGGAGGACACAGTCCGACCATAACAAAC-3'	pTH-2 *Sal*I/*Nco*I
pTH2-CKI3	pRT101-*CKI3*	5'-GCCTCGAGAAAATGCCGGAGTTACGAAAGGG-3' 5'-GCGTCGACACTCATAGTCCGTCCATAGCAG-3' *Xho*I/*Sal*I	pTH-2 *Sal*I
pTH2-CKI4	pRT101-*CKI4*	5'-GCGTCGACAAAATGCCGGTGCTGCGTAACGG-3' 5'-GCGTCGACTGACACTGTGCGACCATAAC-3' *Sal*I partial digestion	pTH-2 *Sal*I
pTH2BN-CKI1	pRT101-*CKI1*	5'-GCCTCGAGAAAATGCCAGAGCTTCGTAGTGG-3' 5'-GAACTAGTCAGCACACCGTGCGTCCGTAAC-3'	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI2	pRT101-*CKI2*	5'-GCGTCGACAAAATGCCTCAACTGCGTAGCGGAG-3' 5'-GAACTAGTCAGGACACAGTCCGACCATAAC-3' *Sal*I/*Spe*I	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI3	pRT101-*CKI3*	5'-GCCTCGAGAAAATGCCGGAGTTACGAAAGGG-3' 5'-GATCTAGATCAACTCATAGTCCGTCCATAG-3' *Xho*I/*Xba*I	pTH-2BN *Xho*I/*Spe*I
pTH2BN-CKI4	pRT101-*CKI4*	5'-GAGAATTCATGCCGGTGCTGCGTAACGG-3' 5'-GAACTAGTCTATGACACTGTGCGACCATAAC-3'	pTH-2BN *Eco*RI/*Spe*I

Summary

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* (Madagascar periwinkle) is a medicinal plant that produces more than 130 monoterpenoid indole alkaloids (MIAs) including the valuable anticancer compounds vincristine and vinblastine. These compounds are rare in *C. roseus* making their extraction for pharmacological use expensive. The chemical structure of these compounds is complex, therefore, they cannot be synthesized in a cost-effective manner. This, stimulated research on MIA biosynthesis worldwide in the past few decades. Jasmonates (JAs) are plant hormones that play an important role in the regulation of the production of MIAs in *C. roseus* (Memelink, 2009). All known biosynthesis genes involved in MIA production are induced by JAs (Van der Fits and Memelink, 2000; Miettinen et al., 2014). Additionally, JAs trigger 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.

The APETALA2/Ethylene Response Factor (AP2/ERF) transcription factors (TFs) Octadecanoid-Responsive *Catharanthus* AP2-domain protein 2 (ORCA2) and ORCA3, are key regulators of several MIA pathway genes including *strictosidine synthase* (*STR*). The genes encoding the ORCA TFs also respond to JAs. The basic Helix-Loop-Helix (bHLH) TF CrMYC2 has been shown to act upstream of ORCA2 and ORCA3, and directly activates their transcription in response to JAs. CrMYC2 is regulated by the Jasmonate ZIM-domain (JAZ) repressors. The JAZ/MYC2 regulatory module is widely conserved among the plant kingdom (Chini et al., 2016; Wasternack and Strnad, 2019). In the absence of the bioactive JAs JA-isoleucine (JA-Ile) the activity of positive regulators of the JAs response, such as CrMYC2, is blocked by the interaction with JAZ proteins. JA-Ile promotes the interaction between JAZs and the F-box protein Coronatine Insensitive1 (COI1), leading to JAZ degradation via the ubiquitin-proteasome pathway and consequently de-repression of TFs including CrMYC2, which can then activate the transcription of their target genes. Some of the *JAZ* genes are target genes of CrMYC2, creating a negative feedback loop. The mutant CrMYC2^{D126N} has a mutation which abolishes the interaction between CrMYC2 and certain members of the CrJAZ family (Goossens et al., 2015; Schweizer et al., 2018). Therefore this mutant is more active because it suffers to a lesser degree from the negative feedback loop.

Controlling biological processes in the cell naturally relies significantly on the regulation of TF activity. Cells modulate TF activity by interaction with other regulatory proteins, such as in the case of the CrMYC2-JAZ module, and by posttranslational modifications such as ubiquitination or phosphorylation that affect TF stability or activity, such as in the case of the JAZ repressors.

The research described in the thesis aimed to study the regulation of the activity of TFs that regulate the MIA pathway, focusing on CrMYC2, ORCA2, and ORCA3.

Chapter 1 gives an overview of MIA production by *C. roseus* and transcription factors used in this thesis work that regulate MIA biosynthesis genes.

In **Chapter 2** studies are described that investigate whether overexpression of the engineered derepressed CrMYC2^{D126N} mutant enhances the production of MIAs. It was possible to obtain cell lines overexpressing wild-type CrMYC2. However, in these cell lines expression of MIA

biosynthesis genes was unchanged, probably due to the negative feedback loop. We did not succeed in obtaining cell lines overexpressing CrMYC2^{D126N} even when using the estradiol-inducible XVE expression system. This indicates that overexpression of the CrMYC2 mutant is probably toxic, and that the XVE system is leaky. Due to these technical drawbacks we were unable to answer our research question.

In an attempt to isolate regulators of the ORCA TFs we performed protein-protein interaction screenings based on the yeast two-hybrid system. In **Chapter 3** we describe studies on the F-box protein O2.51 which was found as an interactor of ORCA2. We wanted to test the hypothesis that ORCA2 and possibly ORCA3 are targeted for degradation by this F-box protein. One approach was to generate stably transformed cell lines with overexpression or silencing of O2.51. However, the cell lines we obtained showed either no alterations in the O2.51 expression level or showed variable gene expression patterns making reliable conclusions impossible. In transient trans-activation assays O2.51 inhibited the activities of ORCA2 and ORCA3, a result that is compatible with our working hypothesis. To prove the hypothesis however further experiments are needed.

In **Chapter 4**, we describe studies on members of a small family of four casein kinase I proteins that were found as ORCA3 interactors. CKI is a Ser/Thr protein kinase that works as a monomer. The CrCKIs belong to a plant-specific group of CKIs with members that are involved in various developmental and signal transduction processes. One approach was to generate stably transformed cell lines with overexpression of CKIs. However among the many cell lines we generated none overexpressed CKI. Kinase studies showed that the CKIs were able to phosphorylate the ORCAs. An inhibitory Ser-rich domain in ORCA3 was a major phosphorylation site, suggesting that phosphorylation by CKI is inhibitory. Consistent with this notion, co-expression of CKI in trans-activation assays strongly inhibited the activities of ORCA2 and ORCA3. Cellular localization studies using CKI-GFP fusions showed that the CKIs are located in the nucleus of *C. roseus* cells, consistent with a possible role in TF phosphorylation. The results suggest that ORCA activity in *C. roseus* is attenuated by phosphorylation by CKI. Knocking out CKI genes or use of non-phosphorylatable ORCAs may be approaches to enhance expression of ORCA target genes and possibly MIA production. In a speculative but attractive model the ORCA interactors O2.51 and CKI may work together. Maybe phosphorylation by CKI enhances the affinity of the ORCAs for O2.51 leading to enhanced degradation. This model may be tested by combining ORCAs, O2.51 and CKI in transactivation assays, or by performing yeast tri-hybrid assays.

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Samenvatting

Planten produceren een groot aantal secundaire metabolieten, waaronder terpenoïden, fenolische verbindingen en alkaloïden. Deze verbindingen zijn essentieel voor interacties tussen planten en hun omgeving, en een aanzienlijk aantal ervan heeft farmacologische activiteit bij mensen. *Catharanthus roseus* (roze maagdenpalm) is een medicinale plant die meer dan 130 monoterpenoïde indool alkaloïden (MIAs) kan produceren, waaronder de waardevolle antikankerverbindingen vincristine en vinblastine. Deze verbindingen komen in lage concentraties voor in *C. roseus*, wat hun extractie voor farmacologisch gebruik kostbaar maakt. De chemische structuur van deze verbindingen is complex, waardoor ze niet op een kosteneffectieve manier kunnen worden gesynthetiseerd. Dit heeft in de afgelopen decennia wereldwijd onderzoek naar MIA-biosynthese in *C. roseus* gestimuleerd.

Jasmonaten (JAs) zijn plantenhormonen die een belangrijke rol spelen bij de regulatie van de productie van MIAs in *C. roseus* (Memelink, 2009). Alle bekende biosynthesegenen betrokken bij MIA-productie worden geïnduceerd door JAs (Van der Fits en Memelink, 2000; Miettinen et al., 2014). Bovendien activeren JAs genexpressie in de primaire stofwisseling, wat leidt tot de vorming van MIA-bouwstenen, wat aangeeft dat JAs een sterke invloed hebben op genexpressie en stofwisseling in planten.

De APETALA2/Ethyleenresponsfactor (AP2/ERF) transcriptiefactoren (TFs) Octadecanoïde-responsieve *Catharanthus* AP2-domeineiwitten 2 (ORCA2) en ORCA3 zijn sleutelregulatoren van verschillende MIA-biosynthesegenen, waaronder strictosidine synthase (STR). De genen die coderen voor de ORCA TFs reageren ook op JAs. De basische Helix-Loop-Helix (bHLH) TF CrMYC2 blijkt upstream van ORCA2 en ORCA3 te werken en activeert direct hun transcriptie als reactie op JAs. CrMYC2 wordt gereguleerd door de Jasmonaat ZIM-domein (JAZ) repressoren. De JAZ/MYC2-regulatiemodule is breed geconserveerd in het plantenrijk (Chini et al., 2016; Wasternack en Strnad, 2019). Bij afwezigheid van de bioactieve JAs JA-isoleucine (JA-Ile) wordt de activiteit van positieve regulatoren van de JAs-respons, zoals CrMYC2, geblokkeerd door de interactie met JAZ-eiwitten. JA-Ile bevordert de interactie tussen JAZs en het F-box-eiwit Coronatine Insensitive1 (COI1), wat leidt tot afbraak van JAZ via de ubiquitine-proteasoomroute en daarmee de repressie opheft van TFs zoals CrMYC2, die vervolgens de transcriptie van hun doelwitgenen kunnen activeren. Sommige JAZ-genen zijn doelwitgenen van CrMYC2, wat een negatieve feedback-lus creëert. De mutant CrMYC2D126N heeft een mutatie die de interactie tussen CrMYC2 en bepaalde leden van de CrJAZ-familie voorkomt (Goossens et al., 2015, Schweizer et al., 2018). Hierdoor is deze mutant actiever omdat deze in mindere mate onderhevig is aan feedback-inhibitie.

Het beheersen van biologische processen in de cel is van nature sterk afhankelijk van de regulatie van TF-activiteit. Cellen moduleren TF-activiteit door interactie met andere regulatoire eiwitten, zoals in het geval van de CrMYC2-JAZ-module, en door post-translationele modificaties zoals ubiquitinering of fosforylering die de stabiliteit of activiteit van TF beïnvloeden, zoals in het geval van de JAZ-repressoren.

Het in dit proefschrift beschreven onderzoek had als doel de regulatie van de activiteit van TFs te bestuderen die de MIA-route reguleren, met een focus op CrMYC2, ORCA2 en ORCA3.

- **Hoofdstuk 1** geeft een overzicht van MIA-productie door *C. roseus* en de transcriptiefactoren in de MIA-biosynthese die in dit proefschrift worden bestudeerd.

- In **Hoofdstuk 2** worden studies beschreven die onderzoeken of overexpressie van de gemodificeerde, ge-de-represseerde CrMYC2D126N mutant de productie van MIAs verhoogt. Het was mogelijk om cellijnen te verkrijgen met overexpressie van wild-type CrMYC2. Echter, in deze cellijnen was de expressie van MIA-biosynthesegenen onveranderd, waarschijnlijk door de negatieve feedback-lus. We slaagden er niet in cellijnen met overexpressie van CrMYC2D126N te verkrijgen, zelfs niet met het estradiol-induceerbare XVE-expressiesysteem. Dit wijst erop dat overexpressie van de CrMYC2D126N mutant waarschijnlijk toxisch is en dat het XVE-systeem lek is. Door deze technische beperkingen konden we onze onderzoeksvraag niet beantwoorden.
- In een poging om regulatoren van de ORCA TFs te isoleren, voerden we eiwit-eiwit-interactie screenings uit op basis van het gist-twee-hybridesysteem. In **Hoofdstuk 3** beschrijven we studies over het F-box-eiwit O2.51, dat werd geïdentificeerd als een interactor van ORCA2. We wilden de hypothese testen dat ORCA2 en mogelijk ORCA3 worden afgebroken via herkenning door dit F-box-eiwit. Eén benadering was om stabiel getransformeerde cellijnen te genereren met overexpressie of silencing van O2.51. De verkregen cellijnen vertoonden echter geen veranderingen in het O2.51-expressieniveau of vertoonden variabele genexpressiepatronen, waardoor geen betrouwbare conclusies getrokken konden worden. In transiënte transactivatie-assays remde O2.51 de activiteit van ORCA2 en ORCA3, een resultaat dat compatibel is met onze hypothese. Om de hypothese te bewijzen zijn echter verdere experimenten nodig.
- In **Hoofdstuk 4** beschrijven we studies over leden van een kleine familie van vier caseïnekinase I-eiwitten (CKIs) die als ORCA3-interactoren werden geïdentificeerd. CKI is een Ser/Thr eiwitkinase dat als monomeer werkt. De CrCKIs behoren tot een plant-specifieke groep CKIs waarvan leden betrokken zijn bij verschillende ontwikkelings- en signaaltransductie-processen. Pogingen om stabiel getransformeerde cellijnen te genereren met overexpressie van CKIs waren onsuccesvol. Kinase-studies toonden aan dat de CKIs de ORCAs konden fosforyleren. Een inhiberend Ser-rijk domein in ORCA3 was een belangrijk fosforylatiegebied, wat suggereert dat fosforylering door CKI inhiberend werkt. Consistent hiermee remde co-expressie van CKI in transactivatie-assays sterk de activiteit van ORCA2 en ORCA3. Lokalisatiestudies met CKI-GFP-fusies toonden aan dat de CKIs zich in de kern van *C. roseus* cellen bevinden, consistent met een mogelijke rol in TF fosforylering. De resultaten suggereren dat de activiteit van ORCAs in *C. roseus* wordt verzwakt door fosforylering door CKI. Het uitschakelen van CKI genen of het gebruik van niet-fosforyleerbare ORCAs kunnen benaderingen zijn om de expressie van ORCA doelwitgenen en mogelijk MIA-productie te verhogen. In een speculatief maar aantrekkelijk model zouden de ORCA-interactoren O2.51 en CKI kunnen samenwerken. Mogelijk verhoogt fosforylering door CKI de affiniteit van de ORCAs voor O2.51, wat leidt tot verhoogde afbraak. Dit model kan worden getest door ORCAs, O2.51 en CKI te combineren in transactivatie-assays, of door gist-tri-hybride assays uit te voeren.

Curriculum Vitae

Mina Darehei was born on June 3rd, 1989, in Hamedan, Iran. After graduating from the Fatimeh-Zahra high school at Hamedan in 2006, she studied agriculture engineering- horticulture at the faculty of agriculture, of the Bu-Ali Sina university (Hamedan, Iran). She continued her master studies in medicinal plants at Bu-Ali Sina university. Her master thesis focused on identification of genetic diversity in seven populations of *Stachys lavandulifolia* based on ISSR molecular markers. In 2016, she was awarded a PhD scholarship by the Iranian Ministry of Science, Research, and Technology. She was a lecturer in Bu-Ali Sina university during 2017. In September 2018, she started her PhD research under supervision of Prof. Dr. Johan Memelink (Plant Cell Physiology) at the institute of Biology Leiden (IBL) of Leiden University (The Netherlands). She studied the transcriptional regulation of monoterpenoid indole alkaloid in *Catharanthus roseus* that is described in this thesis.

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Mina Darehei, Leiden
January 2025