Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus

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Chapter 2

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

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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.

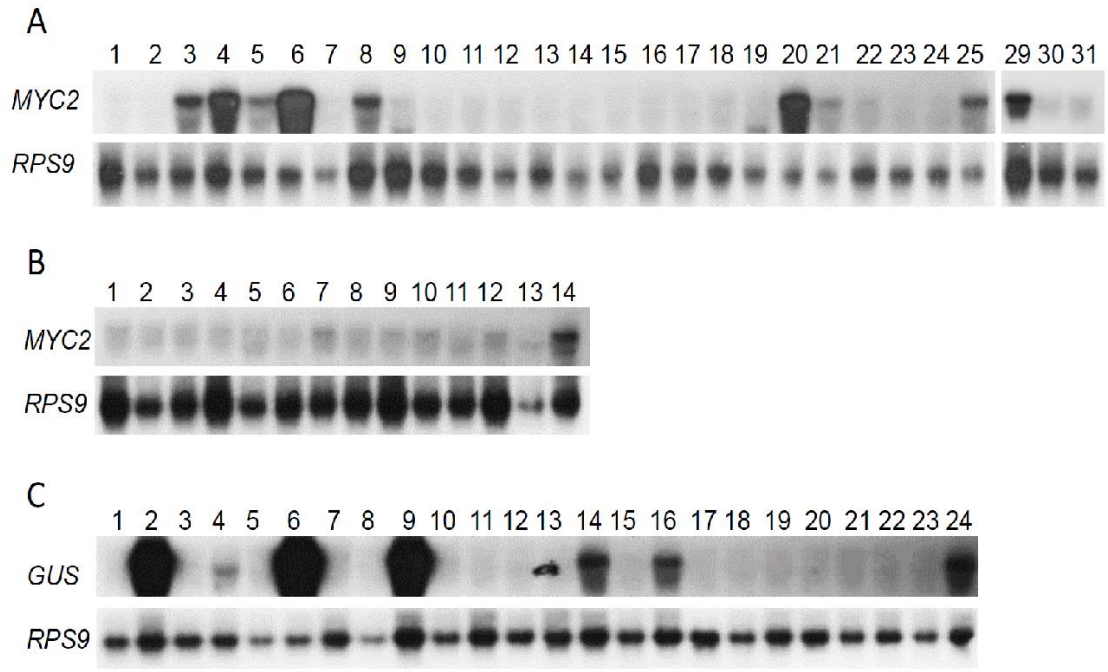

Figure 1. Analysis of transgene expression levels in stable hyg^R cell lines by RNA gel blot hybridisation. The generated cell lines were treated with 10 µM estradiol for 24 hours and analysed for overexpression of the bombarded genes. (A) Cell lines bombarded with wild-type *CrMYC2a* in plasmid pER8 (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).

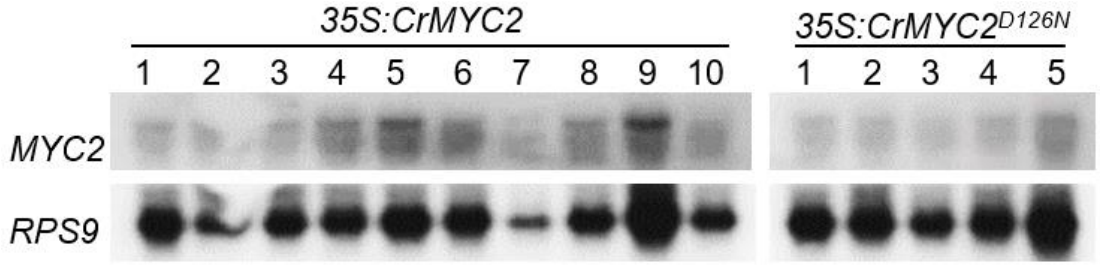

Figure 2. Analysis of the cell lines obtained by bombardment with *35S:CrMYC2a* and *35S:CrMYC2a*^{D126N}. RNA gel blots were hybridized with radio-labeled *CrMYC2a* or *RPS9* as a control.

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.

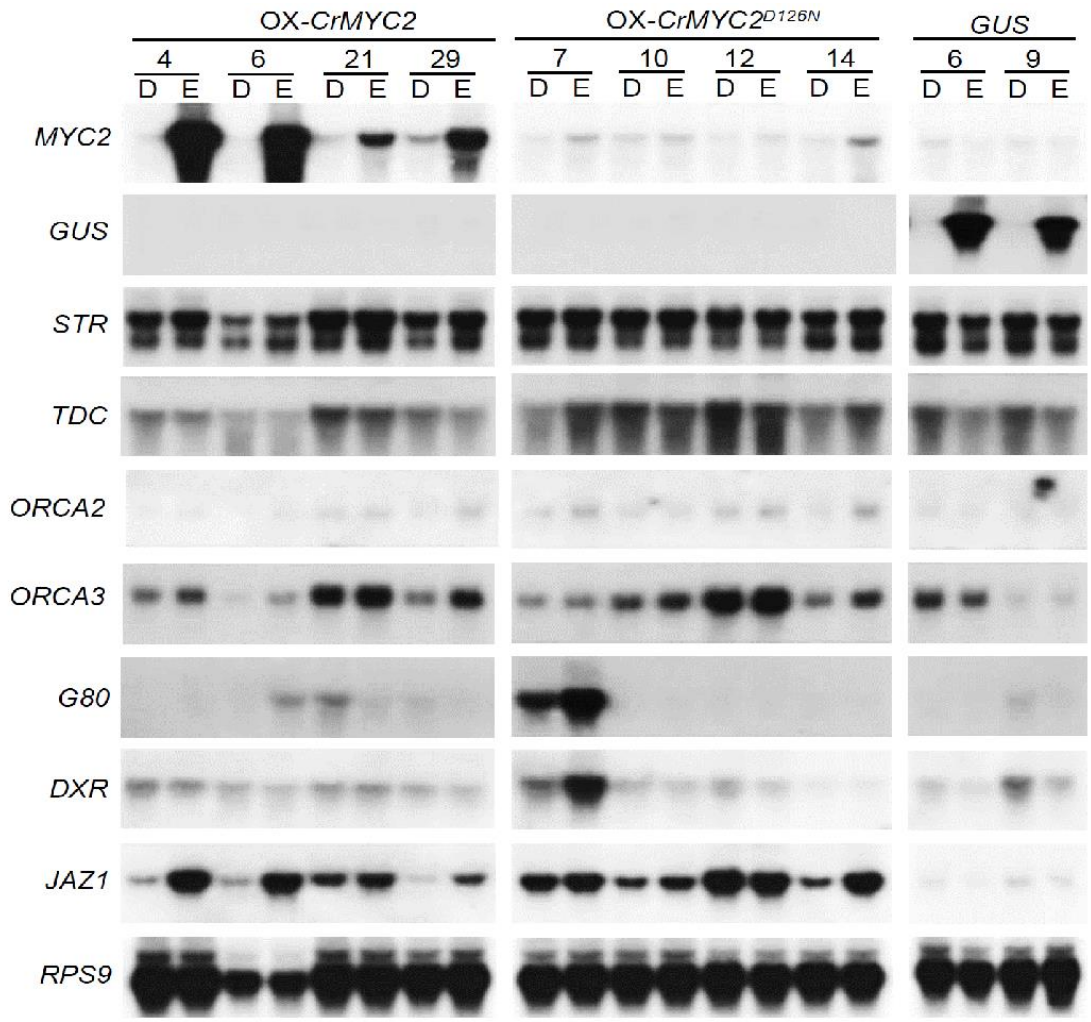

Figure 3. Analysis of the expression of TF genes and biosynthesis genes from the MIA pathway. Selected cell lines were treated with 0.1% DMSO (D) or 10 µM estradiol (E) for 24 hours. The extracted RNAs were analysed by RNA gel blot hybridization for the expression levels of different genes using *RPS9* 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.

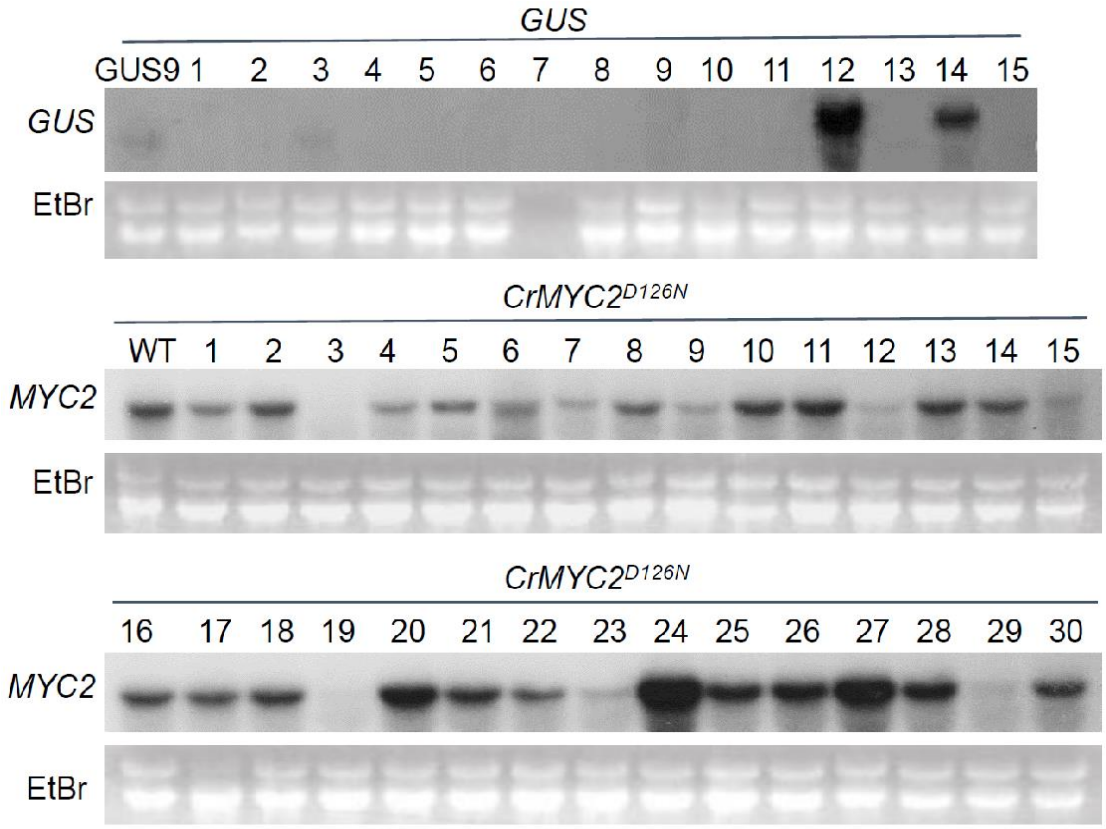

Figure 4. Expression analysis of *C. roseus* cell lines generated following particle bombardment with *CrMYC2a*^{D126N} in pER8. The generated cell lines were treated with 10 µM estradiol for 24 hours and analysed for overexpression of the bombarded genes. Northern blots containing identical amounts 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.

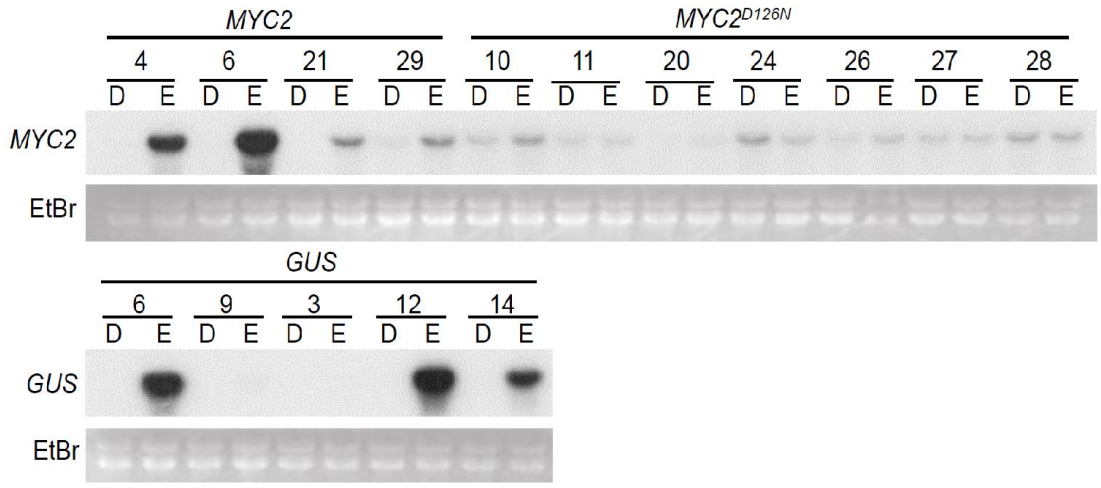

Figure 5. Analysis of transgene expression levels in stable hyg^R cell lines. Genes were in plasmid pER8. The cell lines were treated either with 10 µM estradiol (E) or 0.1% DMSO (D) for 24 hours and analysed for transgene expression levels by RNA gel blot hybridization. Genes bombarded are shown on 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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