Transcriptional regulation of monoterpenoid indole alkaloid biosynthesis in Catharanthus roseus

Darehei, M.

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.

References

Chini A, Gimenez-Ibanez S, Goossens A, Solano R (2016) Redundancy and specificity in jasmonate signaling. Curr Opin Plant Biol 33: 147-156

Goossens J, Swinnen G, Vanden Bossche R, Pauwels L, Goossens A (2015) Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity. New Phytol 206: 1229-1237

Memelink J (2009) Regulation of gene expression by jasmonate hormones. Phytochem 70: 1560-1570

Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, Van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus*. Nature Commun 5: 3606

Schweizer F, Colinas M, Pollier J, Van Moerkercke A, Vanden Bossche R, De Clercq R, Goossens A (2018) An engineered combinatorial module of transcription factors boosts production of monoterpenoid indole alkaloids in *Catharanthus roseus*. Metabol Eng 48: 150-162

Van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297

Wasternack C, Strnad M (2019) Jasmonates are signals in the biosynthesis of secondary metabolites - Pathways, transcription factors and applied aspects - A brief review. New Biotechnol 48: 1-11