DISSERTATION

METABOLIC ENGINEERING AND ELUCIDATION OF THE TERPENOID INDOLE ALKALOID PATHWAY IN *CATHARANTHUS ROSEUS* HAIRY ROOTS

Submitted by

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ABSTRACT

METABOLIC ENGINEERING AND ELUCIDATION OF THE TERPENOID INDOLE ALKALOID PATHWAY IN *CATHARANTHUS ROSEUS* HAIRY ROOTS

Catharanthus roseus (*Madagascar periwinkle*) produces many pharmaceutically important chemicals such as vinblastine, vincristine, serpentine, and ajmalicine. They are synthesized through the highly branched and complex terpenoid indole alkaloids (TIA) pathway in *C. roseus.* Among these TIAs, vinblastine and vinblastine, which are solely extracted from *C. roseus*, are the efficient anti-cancer drugs widely used in the clinic. However, due to the low accumulation of these TIAs within the plant and the industrial infeasibility of production using chemical synthesis, the market price of these drugs still remain high, and the production is inconsistent. With the advanced knowledge of molecular biology, metabolic engineering and bioinformatics, building a robust and efficient alternative production platform by manipulating the TIA pathway has become a major trend and promising strategy in recent research.

However, many biosynthetic enzymes in TIA pathway and the regulation of the pathway are still poorly understood which impedes the rational engineering of this plant for enhanced TIA production. This thesis first uses advanced high-throughput sequencing technology to study the global transcriptional alterations after overexpressing a rate-limiting enzyme anthranilate synthase (AS) in the pathway. This study helps to increase understanding of TIA regulation in this transgenic hairy root line from a broader perspective. Furthermore, transcriptome

sequencing of this unique transgenic line under three different conditions (uninduced control, induced AS overexpression, and methyl jasmonate elicitation) is analyzed using hierarchical clustering. A 200 candidate transcripts set was identified for the pathway genes located around the tabersonine branch point. Six cytochrome P450 monooxygenase candidates are selected for the unknown tabersonine 6,7 epoxidase that can convert tabersonine to lochnericine in *C. roseus* hairy roots.

Meanwhile, effort on genetic modification of *C. roseus* hairy roots for TIA production using two different strategies are reported here. The first strategy helps establish a transgenic hairy root line with significantly increased TIA accumulation of all measure alkaloids by co-expressing the positive transcription factor ORCA3 (AP2-domain DNA-binding protein 3) and a pathway gene strictosidine glucosidase (SGD) that is not controlled by ORCA3. Since *C. roseus* hairy roots do not produce detectable vinblastine and vincristine due to the absence of the vindoline pathway, the second strategy initiated the effort to introduce the pathway by engineering the first two enzymes in *C. roseus* hairy root. Overexpression of these two genes, tabersonine 16-hydroxylase (*T16H*) and 16-O-methyl transferase (*16OMT*), leads to the accumulation of the expected vindoline pathway intermediates 16-hydroxytabersonine and 16-methoxytabersonine but not vindoline. Interestingly, the overexpression of these two genes influences the root native metabolite levels, triggers the altered transcription of TIA genes, and leads to the production of two new unknown metabolites.

Overall, studies in this thesis not only contribute new transcriptome information to current publicly available databases, but also facilitate elucidating the TIA pathway and its complex

regulation. This thesis also provides a metabolic engineering approach to enhance alkaloid production in *C. roseus* hairy roots by simultaneously overexpressing *ORCA3* and *SGD*. Genetic modification of *T16H* and *16OMT* in *C. roseus* hairy roots promisingly leads to the production of vindoline pathway intermediates. It also emphasizes some potential complexities for the future attempts to express the full vindoline pathway in hairy roots.

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CHAPTER 1. CONCLUSIONS

There are a number of positive outcomes presented in the results of the preceding thesis. First, based on the fact that TIA biosynthesis is a tightly coordinated process, and genetic modifications of this pathway tend to be limited in the increases in TIA accumulations, a comprehensive transcriptomic analysis of *C. roseus* hairy roots overexpressing the rate-limiting enzyme AS in the indole pathway was reported to understand the complex regulation of the TIA pathway. The results showed overexpressing *AS* stimulated the overall stress response and broadly influenced the metabolic network and multiple signal transduction pathways. The up-regulation of endogenous JA biosynthesis pathway provide the direct reason why many of the transcripts for TIA genes and regulators are seen to increase with *AS* overexpression [1].

Second, a co-expression analysis of the transcriptome from three differently treated AS transgenic hairy roots was described in this thesis to provide candidates for the unknown TIA genes close to tabersonine branch. Around 200 transcripts showing up-regulation in methyl jasmonate (MeJA) elicited hairy roots and down regulation in *AS* overexpressing hairy roots were clustered with tabersonine 19-hydroxylase (T19H), minovincinine 19-hydroxy-O-acetyltransferase (MAT), and SGD genes. By further searching for annotated cytochrome P450 monooxygenase transcripts, four full-length candidate genes were selected for the un-elucidated tabersonine 6,7-epioxidase which catalyzes tabersonine to lochnericine.

Third, the eleven years stability of AS transgenic line was demonstrated by evaluating the enzyme activity of AS and the terpenoid indole alkaloid metabolite levels in response to the inducible expression. It demonstrated the significant chemical and genetic stability information for the future industry application.

Fourth, a metabolic engineering strategy of co-overexpressing the TIA pathway positive transcription factor *ORCA3* with the pathway gene *SGD* that showed down regulation in the *ORCA3* engineered hairy root successfully enabled the significant increased production of all the measured alkaloids and the overall TIA metabolites in present thesis [2].

Lastly, a transgenic hairy root line with the modification of the first two genes T16H and 16OMT in the vindoline pathway initiated the channeling of tabersonine to vindoline intermediates biosynthesis which is not present in native hairy roots. Besides seeing the accumulation of the expected product of T16H and 16OMT, two additional alkaloids were produced, and the concentration of other root specific metabolites were significantly changed. This study also illustrates how the introduction of T16H and 16OMT triggered complex transcriptional responses, especially the up-regulation of negative transcription factors.

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