CHAPTER VI. CONCLUSIONS AND FUTURE WORK

6.1. A new tool for tuning protein expression

Chapter II describes a novel tool for tuning protein expression levels in yeast. By engineering strains with altered expression of the Gal2p permease, we demonstrated a modified induction response from the native GAL1-10 promoter. Specifically, the *GAL2Δ* strain resulted in a linear and homogeneous response such that protein levels could be finely tuned with galactose concentration.

We demonstrated the utility of this system in our yeast strains engineered for the production of benzylisoquinoline alkaloids (BIAs). By titrating one of three enzymes in a short heterologous pathway independently, we were able to determine optimal expression levels of each enzyme. We then selected a constitutive promoter from a well-characterized promoter library[5] that exhibited the desired expression level and made the appropriate substitutions. Constitutive promoters are preferred for this work to avoid the additional induction step and the use of costly inducers. We also observed greater conversion in the constitutively-expressing strains, likely due to the elimination of an uninduced population.

We propose this as a general strategy that can be useful for adjusting protein expression levels for pathway engineering. Although it is not applicable to all systems; for instance, the P450 activities were not tunable in this range, it can greatly simplify the promoter screening and selection process. In addition, this tuning strategy is applicable to many other areas of synthetic biology such as the construction of genetic circuits.

6.2. *De novo* biosynthesis of BIA backbone molecules

While we were able to make significant progress engineering the downstream BIA pathway starting with norlaudanosoline, we ultimately want to synthesize BIA metabolites *de novo* from two molecules of tyrosine. Since this early pathway has not been fully elucidated in plants, we took a bioprospecting approach and combined enzymatic activities from plants, bacteria, humans, and yeast to engineer a unique pathway for the production of norcoclaurine and norlaudanosoline.

We have developed two different synthesis routes for the production of dopamine and 4-hydroxyphenylacetaldehyde (4-HPA) and validated enzymatic activities for each step. However, we are only able to produce very low levels of dopamine and more optimization is required to reach levels sufficient to build the BIA backbone. This may require protein engineering or evolution of the CYP2D6 enzyme used to convert tyramine to dopamine. Similar to other human P450s, CYP2D6 exhibits low activity on a broad range of substrates as we have also used this activity to convert (*R*)-reticuline to salutaridine. Since dopamine and L-dopa activate the yeast oxidative stress response pathway, a reporter gene such as GFP fused to the FUS1 promoter can potentially be used as a high-throughput screen for increased production of either of these molecules[42].

In addition, we have identified endogenous alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALD) activities that act on the acetaldehyde intermediates 4-HPA and 3,4-DHPA. As single knockouts allowed increased accumulation of norcoclaurine and norlaudanosoline, we are hopeful that combinatorial knockouts will show additive effects.

We have also begun work building protein scaffolds and using leucine zipper domains to co-localize enzymes and increase local metabolite concentrations. These and other strain engineering methods will be required to develop a viable process for the biosynthesis of the BIA backbone.

6.3. Production of the intermediate reticuline and downstream berberine and morphinan alkaloids

We engineered yeast strains for the production of two sets of very diverse BIAs along the berberine and morphinan branches. Currently, we are able to accumulate up to 165 mg l^{-1} of (*R, S*)-reticuline, ~35 mg l^{-1} of (*S*)-tetrahydroberine, and ~15 mg l^{-1} of salutaridinol-7-*O*-acetate. We put considerable effort into optimizing transcriptional activity of the enzymes used to convert norlaudanoline to reticuline. However, intracellular transport is limiting in addition to the affinity of the enzymes for norlaudanosoline as it is not the natural substrate. Total biosynthesis of norcoclaurine *in vivo* will remedy both issues assuming an additional P450 activity CY80B1 can be successfully incorporated.

Along the berberine branch, we are able to accumulate (*S*)-scoulerine, (*S*)-tetrahydrocolumbamine, and (*S*)-tetrahydroberberine. Very recently, a protein with (*S*)-cheilanthifoline synthase activity was cloned from *E. californica*[95]. This enzyme which accepts (*S*)-scoulerine as a substrate will allow access to other metabolites along the sanguinarine branch. In addition, the BIA palmatine can be produced from (*S*)-tetrahydrocolumbamine once the enzyme opening this branch through columbamine is cloned. And finally, (S)-tetrahydroberberine can be oxidized to the final product

berberine in our yeast strains once the (*S*)-tetrahydroberberine oxidase (STOX) activity is cloned from plant hosts or an alternative enzyme is identified. Although we see efficient conversion to (*S*)-tetrahydrocolumbamine, we only see ~10% conversion to (*S*)-tetrahydroberberine which makes this step a target for additional optimization.

Preliminary strains were constructed for production of morphinan alkaloids which leave room for improvement at every step. Once again, we observed low activity from both P450s tested in our system for the production of salutaridine. This is not surprising for the human P450 CYP2D6 but higher activity is expected for the plant enzyme SalSyn (CYP719B). We tested multiple reductase partners here to no avail and feel this may be the limitation in our system. Low accumulation of salutaridine also affects SalR activity as the enzyme is inhibited at low substrate concentrations. Testing of different SalR variants and mutants as well as codon-optimization did not yield significant improvements. SalR is also an NADPH-dependent enzyme so the redox balance of the cell may need to be taken into consideration. For the SalAT step, we were able to significantly improve expression through codon-optimization but are still plagued by low activity. Since this step also requires acetyl-CoA, other metabolic engineering strategies can be applied to increase this cofactor pool if necessary. In addition, the pH and temperature optima for this enzyme are not compatible with yeast physiological conditions. While we can increase the pH to ~7 in buffered media, we cannot operate at pH>8 which is the reported requirement for thebaine production. Ultimately, a 2-stage process will likely be required. Our early attempts to do this entirely *in vivo* were unsuccessful, and it is likely that the final step must be performed *in vitro*. Alternatively,

the cloning of thebaine synthase (THS) from poppy plants and its incorporation into this heterologous pathway may facilitate efficient thebaine production in our yeast hosts.

6.4. Construction of a strain to produce downstream BIAs from tyrosine

The end goal of this work is the construction of yeast strains capable of performing the total biosynthesis of complex BIAs from tyrosine. To reach this goal, much more optimization is required in each segment of the pathway, particularly the upstream portion. We have begun to merge the two pathways by producing reticuline from dopamine and coclaurine from exogenous dopamine and 4-HPA, demonstrating the feasibility of combining these segments. Also, as predicted, conversion is greater when the BIA precursor is synthesized *in vivo* rather than added to the media, avoiding transport limitations. Presuming we can construct a strain to produce norcoclaurine and norlaudanosoline, there is no reason to believe we cannot begin to piece together a stable strain to produce reticuline from tyrosine. From there, plasmid-based expression of downstream enzymes should be sufficient to extend these pathways down various branches. The work described here is significant as it marks the first demonstration of reconstructing this very important pathway in a single microbial host.

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