Metabolic Pathway Engineering of Paclitaxel Biosynthesis in *Taxus chinensis* Plant Cell Culture

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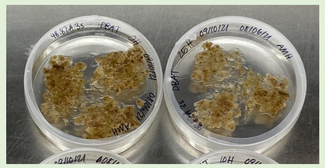
Rationale for metabolic pathway engineering

Paclitaxel, a chemotherapeutic produced by plants in the *Taxus* genus, is a widely used FDA-approved drug for treatment of lung, breast, and ovarian cancer and **one of few compounds industrially produced using plant cell culture[1].**

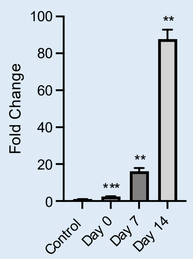
Plant cell culturing is the most suitable method for production of paclitaxel because of its complex structure but it tends to have **low product yield.**

Since other elicitation methods have led to decreased cell growth and viability, this research aims to **increase production of paclitaxel via metabolic pathway engineering.**



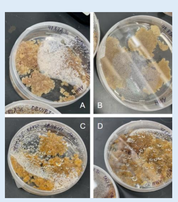
Development of a stable transformation method for *Taxus* PCC

***Taxus chinensis* cell line 48.82A.3s was transformed** with a vector overexpressing the GUS+ reporter gene using a stable *Agrobacterium* infiltration-based transformation method. A **significant increase in expression of the GUS+ reporter gene** (quantified by RT-qPCR) was observed over a 14-day period (right).

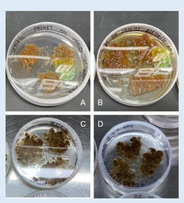


Stable integration of the selectable marker (hygromycin resistance) was achieved **3 months after the initial transformation** of *Taxus* cells and confirmed with a PCR screen of genomic DNA (left).

Successful selection of transformed *Taxus* cells visually confirmed



Non-selective media



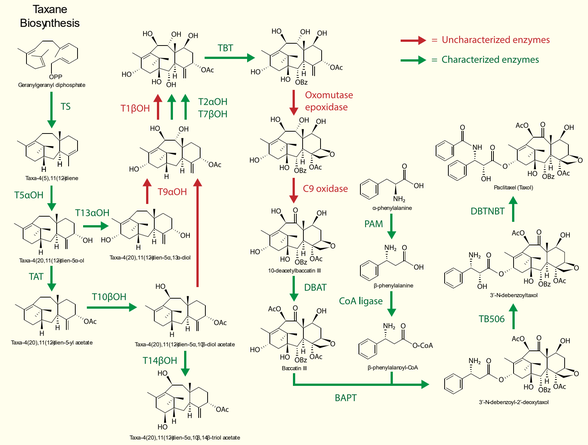
Selective media

(A) DBTNBT
(B) DBAT
(C) BAPT
(D) TASY

A healthy light brown color was observed two weeks after transformation, indicating **successful cell recovery** (above, left).

Darkening of these calluses was then observed when transferred to selective media with 10 µg/mL hygromycin, indicating **successful selection** (above, right)**.**

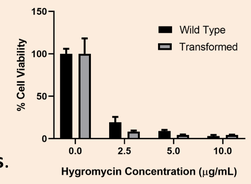
Paclitaxel biosynthetic pathway



Viability assays revealed optimum concentration of hygromycin for selection

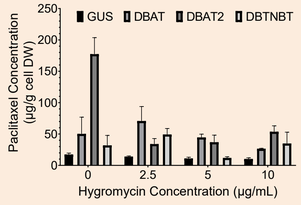
The optimum concentration of hygromycin for selection was determined by measuring cell viability with a resazurin assay.

Hygromycin was found to be effective, killing **>96% of WT cells**.



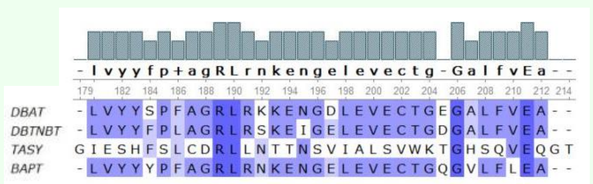
10 µg/mL was the optimum hygromycin concentration as it killed more wild type cells than it did transformed cells.

The **concentration of paclitaxel increases** when DBAT and GUS+ are overexpressed across all hygromycin concentrations. Compared to the control, DBAT2 and DBTNBT were found to be statistically insignificant.



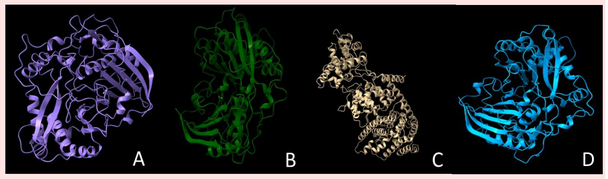
Multiple sequence alignment of *Taxus* biosynthetic pathway enzymes

Multiple sequence alignment (MSA) was used to compare the sequences of the four genes of interest in the paclitaxel biosynthetic pathway. **DBAT, DBTNBT, and BAPT had increased alignment** compared to TASY, reflecting the mechanisms and locations of the enzymes in the biosynthetic pathway.

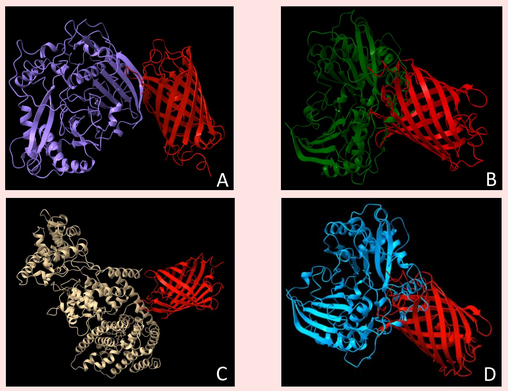


Visualization of mCherry fusion proteins using homology modeling

Homology modeling was performed to predict structures of mCherry fusion proteins with four genes of interest to predict any changes in secondary structure.

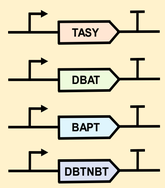


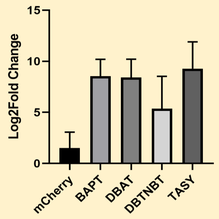
(A) BAPT (B) DBAT (C) TASY (D) DBTNBT



Quantification of gene expression and paclitaxel production

Four vectors overexpressing paclitaxel biosynthetic pathway enzymes were transformed into *Taxus chinensis*.

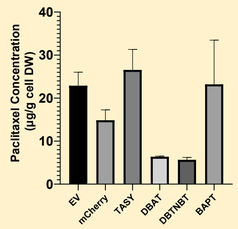


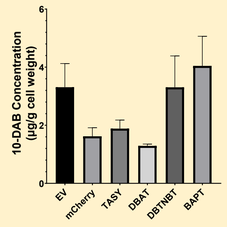


A **large increase in gene expression was confirmed using RT-qPCR** in all but was slightly lower for mCherry.

Paclitaxel and 10-DAB concentrations were quantified using UPLC. A lower concentration of paclitaxel (below, left) was identified after overexpression of DBAT and DBTNBT. This is inconsistent with previous literature, possibly due to incubation time after elicitation being decreased.

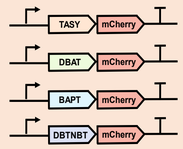
There were **lower concentrations of 10-DAB overall** (below, right) when compared to paclitaxel concentration, indicating efficient conversion of 10-DAB to paclitaxel.





Non-destructive screening using mCherry fusion proteins

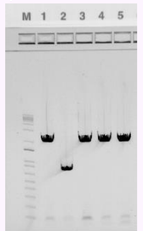
A **non-destructive screening method** was initiated using mCherry-tagged *Taxus* biosynthetic pathway enzymes to more easily generate stable transgenic cell lines. This will also allow us to study **intracellular enzyme localization**.



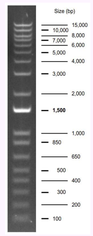
Cloning strategy for mCherry-tagged paclitaxel biosynthetic pathway enzymes

We **successfully constructed mCherry vectors using Gibson assembly** and confirmed transformation into *E.coli* DH5α using colony PCR and gel electrophoresis (below, left).

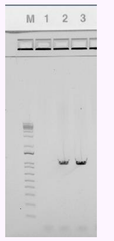
The vectors were then **transformed into *A. tumefaciens* EH105**, which was confirmed using PCR and gel electrophoresis (below, right).



E. coli



DNA Ladder



A. tumefaciens

Conclusions

- Developed a **successful transformation protocol** and **identified a selectable marker** for *Taxus*
- **mCherry vectors were successfully constructed** but a successful transformation into *Taxus* was not completed due to time constraints
- The **transformation with BAPT, DBAT, DBTNBT, and TASY was completed** and analyzed with qPCR and UPLC, which indicated **increased gene expression and increased paclitaxel production**

Future recommendations

- Repeat the transformation without mCherry tag
- Repeat transformation with mCherry-tagged biosynthetic pathway enzymes
  - Confirm increased gene expression and paclitaxel concentration with **qPCR and UPLC**, respectively
  - Perform **flow cytometry** to confirm fluorescence
  - Perform **confocal microscopy** to characterize enzyme localization

References

1. Mutanda, I., Li, J., Xu, F., & Wang, Y. (2021). Recent Advances in Metabolic Engineering, Protein Engineering, and Transcriptome-Guided Insights Toward Synthetic Production of Taxol. Frontiers in Bioengineering and Biotechnology, 9, 632269.
2. Naill MC, Kolewe ME, Roberts SC. Paclitaxel uptake and transport in Taxus cell suspension cultures. Biochem Eng J. 2012 Apr 15;63:50-56. doi: 10.1016/j.bej.2012.01.006. Epub 2012 Jan 29. PMID: 23180977; PMCID: PMC3501751.